Ontogeny, Function and Regulation of Follicular T Cells

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Ontogeny, Function and Regulation of Follicular T Cells

A dissertation presented
by
Waradon Sungnak
to
The Division of Medical Sciences
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy
in the subject of
Immunology

Harvard University
Cambridge, Massachusetts
April 2019
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Ontogeny, Function and Regulation of Follicular T Cells

Abstract

Antibody-mediated immunity plays important roles in effective adaptive immune responses against invading pathogens and its dysregulation could lead to detrimental autoimmune diseases. Follicular helper T (T<sub>FH</sub>) cells and follicular regulatory T (T<sub>FR</sub>) cells are specialized CD4 T cells that shape such antibody responses in the germinal centers of secondary lymphoid organs. Here, we utilized single-cell RNA-seq technology to explore the underlying T<sub>FH</sub>/T<sub>FR</sub> cell biology regarding their ontogeny, function and regulation. First, we identified a novel T<sub>FR</sub> cell effector molecule, Fgl2, which directly regulates T<sub>FH</sub> cells and B cells in a context-dependent and type 2 antibody isotype-specific manner. Fgl2 induces Prdm1 in T<sub>FH</sub> cells, along with a panel of co-inhibitory molecules and its deficiency resulted in defective antigen-specific immune responses and spontaneous development of Lupus-like autoimmunity associated with elevated autoantibodies. Second, through single-cell RNA-seq analysis, we found the presence of Th1-like T<sub>FH</sub> cells in our immunization settings and, using T-bet fate reporter mice, demonstrated the existence of linear differentiation trajectory in which Th1 cells could differentiate into Th1-like T<sub>FH</sub> cells. We also verified the role of STAT3 in such differentiation pathway as its genetic deletion resulted in near complete absence of Th1-like, T<sub>FH</sub> cells that previously express T-bet. Taken
together, we not only identified, characterized and verified a novel $T_{FR}$ effector factor that limit antibody responses and humoral autoimmunity but also revealed ontogenic nature of Th1-like $T_{FH}$ cells. Such findings implicate new potential therapeutic targets that could possibly be intervened in order to improve appropriate humoral effector responses in the context of vaccine development, as well as to better suppress autoreactive reactions in the context of autoimmune diseases.
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Undertaking this PhD dissertation through the work in the past years has truly been a life-changing experience for me and it could not have been possible had it not been for the people who have provided support and guidance, which I am sincerely grateful for.

Firstly, I would like to express my gratitude to my advisor, Vijay Kuchroo, for the continuous support for the research projects, for his patience, motivation and immense knowledge. His mentorship has helped me in all the time of my PhD and I am grateful for the time and energy he has invested in me as he always makes his students a top priority.

I would also like to thank Chao Wang, who has been a mentor in the lab. She has taught me fundamentals of conducting scientific research, as well as how to deal with problems that have arisen throughout the years. I always enjoy her passion in science and her commitment to encourage me to be a better scientist.

My sincere thanks also go to my dissertation advising committee: Shiv Pillai, Arlene Sharpe and Nicholas Haining, for their insightful comments, encouragement and hard questions which have incentivized me to tackle my research problems from various perspectives.

I greatly appreciate the support from the Kuchroo lab members and our collaborators. I feel really lucky for the opportunity to learn from all of them. I also owe many thanks to Mary Collin, who has always provided insightful feedback for all of our manuscripts and this dissertation.

Finally, I am grateful for having amazing family and friends. My parents and my sister have always given me unrequited support throughout my life. Also, my classmates in the Immunology Program are among the most passionate immunologists I have known and they have made my graduate school experience truly memorable.
Attributions

Chapter 2

Allon Wagner and Nir Yosef performed computational analysis for population and single-cell RNA-seq experiments. Monika S. Kowalczyk, Orit Rozenblatt-Rosen and Aviv Regev provided collaboration on all genomic work. Peter T. Sage and Arlene H. Sharpe provided CD28\(^{-}\) mice, as well as insights on T\(_{FH}\)/T\(_{FR}\)/B cell co-culture experiments. Raymond A. Sobel performed histological analysis. Francisco J. Quintana helped designing and performing Lupus antigen microarray. Chao Wang provided guidance and feedback on experimental designs.

Chapter 3

Allon Wagner, Zoë Steier and Nir Yosef performed computational analysis for population and single-cell RNA-seq experiments. Monika S. Kowalczyk, Orit Rozenblatt-Rosen and Aviv Regev helped designing performing single-cell RNA-seq in all platforms. Mathias Pawlak crossed and maintained the T-bet fate-mapping mouse strain used in specified experiments. Chao Wang provided guidance and feedback on experimental designs.
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ANA</td>
<td>Antinuclear antibody</td>
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<tr>
<td>AID</td>
<td>Activation-induced cytidine deaminase</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>ASC</td>
<td>Antibody-secreting cell</td>
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<td>BAFF</td>
<td>B-cell activating factor</td>
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<td>BCL-6</td>
<td>B-cell lymphoma 6</td>
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<td>BCR</td>
<td>B cell receptor</td>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CFA</td>
<td>Complete Freund's Adjuvant</td>
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<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
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<tr>
<td>CSR</td>
<td>Class-switch recombination</td>
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<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte-associated protein 4</td>
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<tr>
<td>CXCL</td>
<td>C-X-C motif chemokine ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>C-X-C motif chemokine receptor</td>
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<tr>
<td>DZ</td>
<td>Dark-zone (in germinal centers)</td>
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<td>dsDNA</td>
<td>Double-stranded DNA</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ELISPOT</td>
<td>Enzyme-linked immunospot</td>
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<td>FcgR</td>
<td>Fc gamma receptors</td>
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<td>FDC</td>
<td>Follicular dendritic cell</td>
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<td>Fgl2</td>
<td>Fibrinogen-like protein 2</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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</tr>
<tr>
<td>HAVCR</td>
<td>Hepatitis A virus cellular receptor</td>
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<td>HDM</td>
<td>House dust mite</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>GATA-3</td>
<td>GATA binding protein 3</td>
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<tr>
<td>GC</td>
<td>Germinal center</td>
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<td>ICOS</td>
<td>Inducible T-cell costimulator</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>Interleukin</td>
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<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activating motif</td>
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<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibitory motif</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>LAG-3</td>
<td>Lymphocyte-activation gene 3</td>
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<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LZ</td>
<td>Light-zone (in germinal centers)</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>MHV</td>
<td>Murine hepatitis virus</td>
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<tr>
<td>NP</td>
<td>4-Hydroxy-3-nitrophenylacetyl hapten</td>
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<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor κ-light-chain-enhancer of activated B cells</td>
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<td>OVA</td>
<td>Ovalbumin</td>
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PD-1  Programmed cell death protein 1
RA  Rheumatoid arthritis
RAG  Recombination-activating gene
RF  Rheumatoid factor
RNA-seq  RNA sequencing
RORγt  Retinoic acid-related orphan nuclear hormone receptor gamma
SARS  Severe acute respiratory syndrome
SHM  Somatic hypermutation
SHP  Src-homology 2 domain-containing phosphatase
SLE  Systemic lupus erythematosus
STAT  Signal transducers and activators of transcription
T-bet  T-box transcription factor
TCR  T cell receptor
T<sub>FH</sub>  Follicular helper T cells
T<sub>FR</sub>  Follicular regulatory T cells
TGF-β  Transforming growth factor beta
Th  Helper T cells
TIGIT  T cell immunoreceptor with Ig and ITIM domains
TIM-3  T-cell immunoglobulin and mucin-domain containing-3
TLS  Tertiary lymphoid structure
TPM  Transcripts per million (unit of transcript expression)
Treg  Regulatory T cells
t-SNE  T-distributed stochastic neighbor embedding
Chapter 1

General Introduction
CD4 T Cell Subset Differentiation

The Tripartite Model: Inducing Cytokines, Master Transcription Factors and Effector Cytokines

Optimal immune responses require effector mechanisms that can prevent host damage from a wide array of infectious organisms. CD4 helper T (Th) cells are key players in governing the appropriate class of immune response so that T cells, innate immune cells and B cell-mediated antibody responses are coordinately activated to eradicate invading pathogens. CD4 Th cells can be categorized into different subsets based on the effector cytokines they produce. In their pioneering discovery, Tim Mossman and Bob Coffman identified Th1 and Th2 subsets (1) based on expression of distinct cytokines. Additional CD4 T cell subsets have since been identified, including Th17 cells, Treg cells and T_{FH} cells (see below). The Th1/Th2 paradigm led not only to the discovery of mechanisms of T cell differentiation but also to the identification of their distinct effector functions. CD4 Th cell differentiation is initiated by cytokines produced by the innate immune system and orchestrated by expression of master transcription factors. IL-12-induced Th1 cells, which produce the signature cytokine IFN-γ and provide immunity against intracellular pathogens, require T-bet as their master transcription factor (2-4). On the other hand, IL-4-induced Th2 cells produce IL-4, IL-5 and IL-13, require GATA-3 as their master transcription factor and play important roles in controlling helminth infection (5, 6). Polarization of naïve T cells into Th1 or Th2 cells by chronic stimulation was suggested to result in terminal differentiation, in that reversion of phenotypes could not be induced (7). Moreover, the production of characteristic cytokines of specific subsets was shown to be stable, corresponding to a well-defined number of cell divisions (8, 9). Such findings have reinforced the idea that
Th1/Th2 fate bifurcation results in committed lineages, which are accompanied by epigenetic changes that confer a fixed, stable phenotype.

The Th1/Th2 dichotomy of Th subsets was further expanded with the discovery of regulatory T cells with Foxp3 as their master transcription factor (10-12). Two major categories of Foxp3+ Treg cells have been identified: the naturally-occurring, thymically-derived Treg cells (tTreg or nTreg cells) and the TGF-β-induced peripheral Treg cells (pTreg or iTreg cells) (13-15). These Foxp3+ T cells suppress effector T cell and B cell responses and regulate innate immune cell activity, thereby maintaining peripheral tolerance and preventing development of organ-specific autoimmunity. Loss of the Treg cell master transcription factor Foxp3 leads to multi-organ autoimmunity both in humans and mice (16, 17).

Although Th1 cells were thought to be key drivers in inducing organ-specific autoimmunity, loss of the Th1 signature cytokine IFN-γ or its receptor did not inhibit development of autoimmunity, but rather resulted in exacerbated disease (18-20). This observation suggested that a subset other than Th1 cells was responsible for inducing such autoimmunity and provided an impetus for the discovery of IL-17 producing Th17 cells. It was predicted that IL-17-producing Th17 cells may be a distinct lineage that is closely related to Th1 cells, but is induced by a distinct set of cytokines (21) from those involved in the induction of Th1 cells. It was later shown that Th17 cells can be differentiated by IL-6 and TGF-β (22-24), leading to the expression of the Th17 master transcription factor RORγt (25-27) and the production of cytokines IL-17A, IL-17F, IL-21 and IL-22 (28-31).

T helper cells have long been known to help B cells to induce antibody class switching and thereby induce preferred classes of antibodies that can fix complement, mediate antibody-dependent cell-mediated cytotoxicity (ADCC) or neutralize offending pathogens (32-35). It was
previously thought that Th cells themselves directly mediated help to B cells in the B cell follicles in secondary lymphoid organs. However, Th cells generated in paracortical areas on their own are not able to migrate to the B cell follicles. A subset of Th cells was later shown to express the chemokine receptor CXCR5 and to migrate to B cell follicles in response to the chemokine CXCL13 and provide help to B cells with in the follicles (36-38). The Th cells that express CXCR5 and provide help to B cells were named T follicular helper cells (T\textsubscript{FH}) and Bcl6 was identified as the master transcription factor for the generation of the T\textsubscript{FH} cells (39-41). The traditional model of T cell differentiation into different subsets consisting of the three major components is summarized in Figure 1.1. There are also growing studies on additional CD4 T cell subsets, including IL-9-producing Th9 cells induced by TGF-β and IL-4 (42, 43) and IL-10 producing type-1 regulatory T (Tr1) cells induced by IL-27 (44-46) although our understanding of these subsets is still in an early stage in comparison to the subsets mentioned earlier.

The growing catalogue of these T cell subsets, however, was largely construed in a simple, lineage-based model in which cytokine-based induction of each subset-specific master transcription factor results in stable expression of distinct effector cytokines. This model has recently been broadened to include details about repressive mechanisms and epigenetic control of T cell fate decisions.
Figure 1.1 The traditional model of T cell differentiation into different subsets is composed of three major components: inducing cytokines, master transcription factors and effector cytokines. For pTreg/iTreg cells, besides IL-10, their functions involve a wide array of regulatory mechanisms. For T_FH cells, IL-6 and IL-21 have been suggested to be important for mouse T_FH differentiation (47) but their roles have not yet been firmly established due to contradictory results (48, 49). TGF-β, in conjunction with IL-12 or IL-23, or a soluble molecule Activin A, was shown to drive human T_FH differentiation while these conditions did not induce mouse T_FH cells (50, 51).

Repressive Mechanisms in Lineage Commitment

To ensure the fidelity of fate commitment, mechanisms that repress development of other cell fates must operate simultaneously to promote unidirectional T cell differentiation. For example, IL-4 induction of the Th2 program was also shown to repress Th1-inducing IL-12 signaling through inhibition of IL-12Rβ2 expression (4). In Th1 cells, T-bet was shown to induce Runx3, which synergizes with T-bet to promote a feedforward loop to induce expression
of IFN-γ while suppressing expression of IL-4 (52, 53). On the other hand, IL-4 dependent GATA-3 represses Runx3-regulated production of IFN-γ in a T-bet-independent fashion (54). For Th17 cells, IFN-γ and IL-4 were shown to inhibit induction of IL-17 (55, 56), whereas the STAT6-induced transcription factor Gfi-1 in Th2 cells inhibits differentiation of Th17 cells and iTreg cells (57, 58). Although TGF-β signaling induces iTreg differentiation, the presence of IL-6 in the context of TGF-β signaling inhibits iTreg differentiation and favors Th17 differentiation (22, 59). The transcription factor Musculin (MSC) was recently shown to suppress the Th2 transcriptional program upon TGF-β signaling to promote unidirectional development of the iTreg phenotype (60). For Th cells, it was shown that Bcl-6 could inhibit other T cell differentiation pathways in multiple ways. Bcl-6 can inhibit other CD4 T cell differentiation programs by antagonizing Prdm1 (encoding BLIMP-1) expression (39). Bcl6 can bind to the promoters of T-bet and RORγt and repress IFN-γ and IL-17 production (41), inhibit RORγt activity (40) and inhibit Gata-3 at a posttranscriptional level (61). Thus, T cells utilize multiple mechanisms by which they can repress alternate T cell fates and promote unidirectional differentiation of appropriate T cell subset.

**Lineage-specific Epigenetic Architecture**

Master transcription factors bind super-enhancers (SEs), which are large clusters of transcriptional enhancers that drive expression of genes that define cell identity (62, 63). Th1, Th2 and Th17 cells were shown to have distinctive SE landscapes based on chromatin remodeler histone acetyl transferase p300 binding, and lineage-specific transcription factors preferentially bind to SE sites linked to genes for inducing cytokine receptors, transcription factors and effector cytokines, suggesting that subset distinction is present at the epigenetic level (64). It was also
shown that the presence of positive chromatin modifications, such as H3K4me3 and negative modifications, such as H3K27me3, largely conform to the patterns of cytokine expression (IFN-γ, IL-4 and IL-17) of specific helper T cell subsets (65). Similar results were also observed in histone acetylation patterns on lineage-specific effector cytokine loci in Th1, Th2 and Th17 cells (66-68).

Master transcription factors themselves, however, were shown to have little influence on the epigenetic landscape globally. RORγt, for example, has a surprisingly small Th17-related regulatory footprint, as RORγt deficiency had limited effects on p300 recruitment and H3K4 methylation. Its lineage-defining effects, however, rely on priming of the epigenetic landscape by BATF and IRF4 along with transcriptional program initiation by STAT3 (69) and such priming molecules are defined as pioneering factors. The requirement for BATF, IRF4 and STAT3, however, is not only limited to Th17 cells, as Tfh cells also require all of these factors (70-72). Similar observations were also made in Th1 and Th2 cells as STAT4 and STAT1, but not T-bet, were shown to be global regulators of active enhancers based on p300 and H3K4me1 profiles in Th1 cells. In addition, overexpression of GATA-3 only partially restored p300 binding sites lost upon STAT6 deletion in Th2 cells (73). AP-1 mediated assembly of IRF4 or IRF8 with BATF was also shown in Th17 and Th2 cells (74). Similarly, in IL-27-induced IL-10-producing type 1 regulatory cell (Tr1) differentiation, IRF1 and BATF were shown to influence chromatin accessibility and, subsequently, the expression of their effector genes (75). Therefore, the presence of these pioneering factors, though possessing a smaller degree of lineage specificity, is required for lineage-specific functions of master transcription factors.

**CD4 T Cell Plasticity versus Functional Heterogeneity**
The Plasticity Concept and Its Potential Problems

The fidelity of CD4 T cell lineage commitment has been challenged as multiple studies have indicated that T cell fates could potentially be redirected, giving rise to the concept of lineage plasticity. This concept of plasticity was used to define infidelity in lineage commitment, leading to changes in phenotypes of differentiated T cells toward those of other Th subsets. For example, in a mouse model of experimental autoimmune encephalomyelitis (EAE), almost all of the CD4 T cells in the spinal cord that produced IFN-γ had previously expressed IL-17A, implying that they might come from Th17 cells (76). Th17 cells were shown to respond to IL-23 or IL-12 to give rise to progeny that shifted their phenotype toward enhanced IFN-γ expression with loss of IL-17A and IL-17F to some extent, which was contingent upon the presence of TGF-β signaling (77). Moreover, transfer experiments in animal models of diabetes and cancer suggested that Th17 cells could attain a Th1-like phenotype (78, 79). Treg cells were also shown to be able to lose Foxp3 expression and acquire the capacity to produce inflammatory cytokines (80, 81). A significant portion of small intestinal Th17 cells, moreover, were shown to express Foxp3 at some stage of development in Foxp3 fate mapping mice (59). In Peyer’s patches, Th17 cells, as defined by an IL-17 fate reporter, acquired a T\textsubscript{FH} phenotype and induced the development of IgA-producing germinal center B cells (82). In the house dust mite (HDM)-induced allergic reaction model, T\textsubscript{FH} cells were shown to differentiate into IL-4 and IL-13 double-producing Th2 cells that accumulated in the lung and recruited eosinophils (83). In addition, LCMV-specific naïve T cells differentiated under Th2 condition in vitro for 5 days were able to co-express GATA-3 and T-bet and produce IFN-γ upon transfer in the setting of LCMV infection (84). Stable T-bet\textsuperscript{+}GATA-3\textsuperscript{+} CD4 T cells were also observed in the context of parasitic infection (85). The capacity to make some effector cytokines was also seen to be shared
in different CD4 T cell subsets. For example, Th17 cells, TFH cells and, to some extent, Th2 cells share the capacity to secrete IL-21, which is dependent on the transcription factor cMAF (86, 87), even though IL-21 appears to have divergent roles in each subset. For example, IL-21 was demonstrated to be an amplification factor in Th17 cell differentiation (88-90), while it is a TFH effector cytokine that drives growth and plasma cell differentiation (48, 49, 91). As plasticity has been used to explain many of these phenotypic alterations in Th cells, whether these cells actually shifted to a different lineage, however, was not clearly addressed.

There is also evidence suggesting the presence of activating epigenetic modifications of the genes encoding lineage-restricted transcription factors even in the lineages in which these factors are not expressed. Unlike expression of cytokine genes, in which the positive modifications H3K4me3 and negative modifications H3K27me3 largely align with the Th1/Th2/Th17 lineages, the genes encoding the regulatory transcription factors like T-bet and Gata3 exhibit positive (H3K4me3) chromatin modifications in multiple CD4 T cell subsets, suggesting they might be poised for expression (65). However, whether those loci were actually accessible and to what extent the accessibility is lineage-specific is still not known. To address these questions, direct chromatin accessibility assays like DNase-seq, FAIRE-seq or ATAC-seq (92) performed on different helper T cell subsets in a comparative manner is required.

While plasticity has been used to explain potential lineage switches, this concept could also be viewed as self-contradictory. If a lineage is supposed to be faithful, then it should not be plastic, or otherwise it would not be a lineage at all. While we can interpret potential changes in Th cell phenotypes as infidelity of fate commitment, it is also possible that defining the subsets themselves based on expression of master transcription factors and a handful of effector cytokines is problematic. For example, the requirement for pioneering factors, which are by
themselves not lineage-defining and are shared by multiple CD4 T cell subsets, is also fundamental for committed differentiation. Given that the commitment to specific lineages requires stepwise changes in a large number of genes, and includes global epigenetic changes that are necessary for the actions of master transcription factors to stably maintain the effector phenotype, reversal of effector phenotypes would potentially face large barriers. Using a single master transcription factor or a signature cytokine to define a lineage, therefore, might be insufficient without considering the lineage-specific global epigenetic context that was shown in many cases to be independent of master transcription factor expression itself.

Shifting fates, however, might be possible if microenvironments change before commitment and there may be an evolutionary need to repurpose a specific clonotype in a changed environment. For example, although Th1 and Th2 cells that had been polarized in vitro with repeated stimulation showed irreversibility, the reversion of in vitro Th1 and Th2 cells to the alternate fate was possible when cytokine environments changed one week after initial polarization (7). In the LCMV viral infection model, a window of reversal capacity between Th1 and T_{FH} cells was shown to exist in vivo before day 3 (93), and a similar window was observed in a malaria infection model where Th1/T_{FH} cells showed global transcriptomic bifurcation around day 4 (94). However, plasticity in this context would have a more limited scope, as these changes would occur prior to lineage commitment. To use the plasticity concept to describe any Th phenotypic changes and/or overlaps, one must, therefore, consider the larger context and distinguish between lineage switching and functional diversification.

**CD4 T Cell Functional Heterogeneity**
Because Th subsets are defined by one or a few signature cytokines, expression of these cytokines in other Th subsets can be interpreted as subset plasticity. For example, T cells that co-express IL-17 and IFN-γ have been observed in vivo in the context of inflammation and autoimmune diseases (95-97). Alternatively, Th cells may be able to acquire functional diversity in the context of lineage commitment. This would allow committed Th cells to respond to additional environmental signals and modify their repertoire of expressed genes, allowing for greater precision and more diversity in immune responses. These acquired functional states would be an extension of the committed lineage, rather than a switch between lineages. There is evidence that T-bet-mediated IFN-γ in IL-17-producing cells marks a Th17 functional state. Mice lacking T-bet, which is required for Th1 differentiation, were shown to be resistant to Th17-cell-mediated EAE, whereas STAT1 deficient mice remained susceptible (98). Also, IL-17-producing Th17 cells were shown to co-express IFN-γ independently of STAT4 signaling, which is also required for Th1 differentiation (99).

Distinct functional states for Th17 cells have been delineated in the context of EAE, in which Th17 cells generated with TGF-β1 and IL-6, although producing IL-17, were not capable of inducing disease without further exposure to IL-23. IL-23 was shown to play a critical role in stabilizing and reinforcing the Th17 phenotype by increasing the expression of IL-23 receptor (IL-23R) and endowing Th17 cells with “pathogenic” effector functions (90, 100-102). Moreover, it was shown that Th17 cells generated by either TGF-β3 and IL-6 or IL-1β, IL-6 and IL-23 are “pathogenic” in the context of EAE and that these “pathogenic” Th17 cells were shown to co-express T-bet and IFN-γ, whereas “non-pathogenic” Th17 cells expressed high levels of cMaf and IL-10 together with IL-17 (102, 103). These “pathogenic” and “non-pathogenic” Th17 cells are phenotypically in parallel with human Th17 cells. In humans,
different Th17 functional states were observed as Th17 producing cells were shown to exist in
two types with distinct effector functions and differentiation requirements: Candida albicans-
specific Th17 cells produced IL-17 and IFN-γ, but no IL-10 while Staphylococcus aureus-
specific Th17 cells produced IL-17 and could produce IL-10 upon re-stimulation (104). In
addition, humans with bi-allelic inborn RORC mutations not only have impaired immunity
against Candida albicans, but also have impaired immunity against Mycobacterium, which is
associated with defective IFN-γ production by CD4⁺CCR6⁺CXCR3⁺ αβ T cells.

Other factors were also shown to regulate Th17 pathogenicity, including SGK1, PROCR
and RBPJ and their disruption altered the pro-inflammatory phenotype and the severity of EAE
(105-107). More recently, with the advent of single-cell RNA-seq technology that allows
interrogation of cellular heterogeneity in a less biased fashion (108-110), heterogeneity in T cell
subsets has been confirmed. Different states of Th17 cells were observed among Th17 cells that
were differentiated in vivo in mice undergoing EAE, as well as among Th17 cells differentiated
in vitro under either “pathogenic” or “non-pathogenic” polarization conditions. These data
demonstrated that not only there are both “pathogenic” and “non-pathogenic” Th17 cells that are
transcriptionally distinct, but that heterogeneity among cells within each type also exists (111).

Functional heterogeneity is also found in regulatory T cells as activated Treg cells can
express transcription factors associated with other Th lineages. This co-expression of
transcription factors from other lineages was shown to functionally specialize Treg cells to
mediate class-specific control of effector cells. For example, under polarization conditions that
include IFN-γ or IL-27, Treg cells can upregulate T-bet and express the Th1-associated
chemokine receptor CXCR3; these Tregs have been shown to be important for limiting Th1-
mediated inflammation (112, 113). Interestingly, either IFN-γ or IL-27 confers Treg cells with
distinct transcriptional profiles. IL27-induced Treg cells secrete IL10 and are important at mucosal sites whereas IFN-γ-induced Treg cells are important in the periphery (113). T-bet expression in activated Treg cells was shown to be highly stable and suppression of Th1 responses by T-bet\(^+\) Treg cells was shown to be mediated by both T-bet-dependent and T-bet-independent mechanisms (114), suggesting that the T-bet-inducing milieu is likely to dictate the phenotype that leads to induction of subset-specific inhibitory functions in Treg cells. Evidence of such selective suppression was also seen with other effector CD4 subsets as Treg-specific deletion of IRF4 (115) and STAT3 (116) resulted in impaired regulation of Th2- and Th17-mediated responses respectively. In addition, Treg cells expressing the coinhibitory molecule TIGIT were shown to selectively inhibit Th1 and Th17 cell responses, but not Th2 cell responses (117). However, unlike in Treg cells expressing T-bet, the roles for Tregs that expressed GATA-3 and ROR\(\gamma\)t were shown to be more complex than simply mediating inhibition of Th2 and Th17 effector T cell responses, respectively, especially in the context of colonic Treg cells. It was shown that colonic ROR\(\gamma\)t\(^+\) and GATA-3\(^+\) Treg cells are two Treg subpopulations with distinct origins and phenotypes important for maintaining balances in the gut microenvironments where microbiome, inflammation, tolerance and tissue repair are involved (118-121).

Co-expression of master transcription factors and/or signature cytokines of alternate lineages, therefore, could either induce a complete lineage change (plasticity model) or elicit new functions that overlay the original committed lineage (heterogeneity model). Such models must consider the shared pioneering factors between T cell fates, and the combinatorial nature of transcriptional regulation, as well as the degree of lineage-specific global epigenetic accommodation and the extent of lineage-specific gene expression. For example, if T-bet is expressed in a Th17-specific epigenetic landscape, its downstream Th1-like effects could be
much more limited than its effects in bona fide Th1 cells. One might also speculate that because functional states require less genetic and epigenetic commitment, reversibility should be easier, although experiments have yet to be done to verify this in a case-by-case basis. The functional heterogeneity described here emphasizes a distinction from the concept of plasticity in the canonical, lineage-switching sense.

**Regulatory Networks of CD4 T Cell Differentiation and Functions**

Recent genome-wide studies have revealed that complex gene regulatory circuits control different combinations of modules of co-expressed genes in cell differentiation in invertebrates (122), stem cell populations (123) and immune cell types (124-126). Such modularity allowed the system’s components to be separated and recombined, resulting in flexibility and variety in use. Thus, it is not surprising that such a paradigm would also encompass Th cell subsets, orchestrating their differentiation, regulation and effector functions. The capacity to detect a large number of genes, if not all, has allowed the construction of regulatory networks in forms of modules of co-expressed genes, which provides a much deeper understanding of CD4 T cell subsets.

**Regulatory Networks in T Cell Differentiation and Regulation**

Gene regulatory circuits consisting of interacting modules of co-expressed genes are found in Th cell differentiation and regulation. Th17 in vitro differentiation, for example, was modelled, based on time-course gene expression profiling integrated with prior knowledge derived from genomic profiles, into 3 consecutive phases corresponding to 3 densely intra-connected networks that require substantial rewiring. The early phase (0-4hrs) corresponds to
modules of gene expression that include pioneering factors like IRF4 and STAT3, after which
the master transcription factor RORγt was expressed upon entering the intermediate phase (4hrs
– 20hrs), followed by effector cytokine gene expression in the late stage (127). Such findings are
consistent with previous data positing the role of global epigenetic preparation to accommodate
the functions of master transcription factors, which leads to the stable expression of effector
cytokines. Illustration of Th17 dynamic regulatory networks is shown in Figure 1.2.

Interestingly, the analysis of networks of co-expressed genes not only demonstrated the
presence of genes that promote lineage differentiation, either by driving lineage-associated genes
or by repressing genes associated with alternative fates, but also showed that two coupled
mutually-antagonistic gene modules positively and negatively regulate Th17 differentiation,
suggesting the presence of intrinsic potential self-regulating mechanisms (127). While the
positively-regulating module encouraged Th17 differentiation and inhibited development of
alternate cell fates (Th1, Th2 and Treg cells), the antagonist module inhibited Th17
differentiation while promoting alternate cell fates. The two modules were in tension with each
other, but the balance favored Th17 differentiation. Such a lineage-antagonizing module on its
own would make no sense in an isolated context and, thus, exhibits the usefulness of network-
scale studies where the context of other interdependent modules is present.
**Figure 1.2** Differentiation of Th17 cells represented as three consecutive regulatory networks of transcription factors that result in expression of genes involved in each stage of differentiation. The early phase involves responsiveness to inducing cytokines and expression of pioneering factors prior to the start of Rorc (encoding Th17 master transcription factor RORγt) expression in the intermediate phase. The late phase involves expression of effector cytokines including IL-17a and expression of IL23R, which is known to stabilize Th17 phenotype. The figure is adapted from Yosef et al., 2013.

**Regulatory Networks in T Cell Effector Functions**

Modules of co-expressed genes were also demonstrated to be important in conferring Th functional diversification. Single-cell RNA-seq of Th17 cells, differentiated either *in vitro* or *in vivo*, revealed functional heterogeneity corresponding to co-expressed genes that could be categorized into two groups: one that co-varies with a known “pro-inflammatory” gene module,
including Th17 cytokines such as IL-17a and CCL20, and another with a known “regulatory” module including regulatory genes such as IL-10, IL-24 and IL-9. A regulator of such functional states, CD5L, was demonstrated to be enriched in in vitro “non-pathogenic” Th17 cells (induced by IL-6 and TGF-β1) but to co-vary with the pro-inflammatory module. While CD5L itself does not affect Th17 differentiation, its loss converted “non-pathogenic” Th17 cells into “pathogenic” cells in the context of EAE by altering their metabolic status. Other identified regulators, including GPR65, TOSO and PLZP, changed the pathogenic phenotype by altering the “pro-inflammatory” vs. “regulatory” modules of Th17 cells (111, 128). Such results suggest that there is another layer of functional modules expressed on top of the lineage commitment. Simplified illustration of the functional modules mentioned above is shown in Figure 1.3.

![Diagram](image)

**Figure 1.3** Simplified schematic of functional modules found in “non-pathogenic” Th17 with the heatmap showing correlation coefficients. Signature proinflammatory genes could be used to for co-variance analysis on the single-cell gene expression profile of Th17 cells polarized under IL-6 + TGF-β1. Genes that are bimodally expressed in “non-pathogenic” Th17 cells have either positive or correlation with the signature proinflammatory genes, suggesting that such aspect of
Th17 heterogeneity could be attributed to two different effector modules of co-expressed genes. The figure is adapted from Gaublomme et al., 2015.

Another example of an effector functional module is, in fact, a gene module expressed in “exhausted” or “dysfunctional” cells. IL-27 was shown to induce IL-10 expression and drive the differentiation of Tr1 cells (44, 129, 130) and expression of TIM-3 and PD-L1 on CD4 and CD8 T cells (131, 132). Through single-cell RNA-seq, a module of co-inhibitory receptors, including PD-1, TIM-3, LAG-3, TIGIT, PROCR and PDLPN, was identified to be co-expressed on exhausted T cells. This module of co-expressed genes was shown to be partly induced by IL-27, leading to its induction orchestrated by transcription factors Prdm1 and cMaf that act cooperatively to induce expression of the “co-inhibitory” gene module on exhausted T cells. It was demonstrated that the module is part of a larger co-inhibitory gene program that is shared by dysfunctional T cells, including both CD4 and CD8 T cells, in multiple physiological and pathological contexts, including in cancer and chronic viral infections (133). Thus, the data suggest that the paradigm of cytokine dependent induction of regulatory transcription factors resulting in effector molecule induction associated with T cell subset lineage differentiation can also apply to expression of functional modules that can be shared across different T cells in appropriate contexts. Interestingly, more recent evidence suggests that dysfunctional T cells in the context of viral infection and cancer are epigenetically stable (134, 135), implying that such a functional state can even behave as a lineage itself.

Lineage and Functional Modularity in Follicular Helper T Cells and Follicular Regulatory T Cells

Follicular Helper T Cell Ontogenic Conundrum
Follicular helper T (T_{FH}) cells are CD4 helper T cells involved in promoting antibody responses by providing help to B cells in histologically distinct sites known as germinal centers (GCs). With T_{FH} cell help in GCs, B cells undergo affinity maturation, class-switch recombination and memory cell differentiation (35). T_{FH} cells express the chemokine receptor CXCR5 that drives their migration to B cell follicles (36-38). The identification of Bcl-6 as a master regulator of T_{FH} cells established them as a distinct T cell lineage (39-41). However, the relationship of T_{FH} cells with other T cell lineages has been a topic of debate. Although it was shown that T_{FH} cells express greatly reduced levels of Th1-, Th2-, or Th17-related transcription factors and cytokines (36, 136), several studies reported that T_{FH} cells, defined by the CXCR5 marker, still expressed cytokines including IFN-γ (39, 137, 138), IL-4 (138-140) and IL-17 (86). Moreover, it was also shown that, Bcl6^{+} lymph node cells isolated from mice immunized with KLH/CFA could simultaneously express IFN-γ or IL-4. Also, both Th1 and Th2 cells, but not Th17 cells, could be differentiated in vitro for 4 days to become T_{FH} cells. However, the expression of Th1/Th2 signature genes in Bcl6^{+} cells was significantly reduced over time as Bcl6 expression increased (141). IL-17 fate reporter-positive cells, nonetheless, were shown to adopt a T_{FH} phenotype in Peyer’s Patches and were capable of inducing IgA-producing germinal center B cells (82). Intriguingly, the reverse direction was also shown to be possible as T_{FH} cells were shown to differentiate into IL-4 and IL-13 double-producing Th2 cells that accumulated in the lung and recruited eosinophils in the house dust mite (HDM)-induced allergic reaction model (83). Interestingly, while some studies showed that transfer of in vitro differentiated Th1, Th2 or Th17 cells were able to support antibody responses (137, 139, 142), others showed that T_{FH} differentiation in vivo did not depend on Th1, Th2 or Th17 differentiation pathways (136). Phenotypic overlaps between T_{FH} cells and other Th cells may be important for T_{FH}-mediated
class-switch recombination to generate antibody isotypes by B cells, as IFN-γ was shown to induce class switching to isotypes like IgG2a in the context of viral infection (143-145) while IL-4 favors IgG1(mouse)/IgE in the context of parasitic infection (138) and IL-17 favors IgG2a and IgG3 (142).

These phenomena in T_{FH} cells raises some interesting questions about the relationship between T_{FH} lineage/gene regulatory modules and those of other T cell lineages since they seem not to be completely antagonistic. In fact, one of the hotly debated issues in T_{FH} biology is whether T_{FH} cells that produce cytokines typical for other CD4 Th subsets in vivo arise from a distinct T_{FH} lineage that acquires other effector modules (lineage model) or from other CD4 lineages that acquire secondary T_{FH} effector modules (linear model). The models are illustrated in Figure 1.4. As discussed earlier in the context of other CD4 T cells, if Th-like T_{FH} cells are derived from a distinct T_{FH} lineage, it would imply the potential presence of T_{FH}-specific global epigenetic and transcriptional programs that would accommodate the functions of T_{FH} master transcription factor Bcl6, together with a limited transcriptional footprint of lineage-specific transcription factors like T-bet, GATA-3 or RORγt. On the other hand, the opposite results are expected if the linear model is true, where T_{FH} cells would have a large transcriptional and epigenetic footprint of Th lineage-specific transcription factors with limited accommodation of the T_{FH} related gene program.
Figure 1.4 Potential ontogenic models of $T_{FH}$ cells that produce cytokines typical for other CD4 subsets. The lineage model posits that those $T_{FH}$ cells are derived from a distinct $T_{FH}$ lineage that acquires effector modules typical in other helper $T$ cells. On the other hand, the linear model posits that those $T_{FH}$ cells arise from other helper $T$ cell lineages that acquire secondary $T_{FH}$ effector modules.

Lineage versus Effector Modules in Follicular Regulatory $T$ Cells

Recently, a CXCR5$^-$Foxp3$^+$ CD4 $T$ cell population was shown to mediate inhibitory effects on germinal center reactions and antibody responses, and thus the cells were defined as $T$ follicular regulatory $T$ ($T_{FR}$) cells (146-148). Moreover, these cells have been shown to limit autoimmunity responses in animal models of influenza infection and Sjogren’s disease (149, 150). Unlike $T_{FH}$ cells, in which Bcl6 and Prdm1 were shown to be antagonistic (39), Bcl6 and Prdm1 are co-expressed in $T_{FR}$ cells. Prdm1 has been previously shown to influence $Treg$
function by inducing an effector regulatory phenotype (151) and expression of co-inhibitory molecules that are highly expressed on Treg cells (133). Expression of the co-inhibitory receptor CTLA-4 on T_{FR} cells was demonstrated to be important for T_{FR} cell effector functions, as the loss of CTLA-4 on T_{FR} cells led to their reduced capacity to suppress antibody responses (152, 153).

T_{FR} cells express Treg-associated genes along with T_{FH}-associated genes (146-148), thus again raising the question of lineage versus effector modules. These pioneering studies all showed, using adoptive transfer experiments, that tTreg/nTreg cells could adopt a follicular phenotype and become T_{FR} cells. TCR repertoire analysis also showed that T_{FH} cells and T_{FR} cells possess distinct TCR repertoires, with T_{FH} cells expressing foreign antigen-responsive TCRs and T_{FR} cells expressing a repertoire more closely resembling that of nTreg cells, supporting the idea that T_{FR} cells are Treg cells that gain follicular effector modules (154). However, it was also shown that under certain conditions, T_{FR} cells could be differentiated from Foxp3- cells and their specificity was specific for immunizing antigen (155), thus complicating our initial understanding of developmental pathways for T_{FR} cells. This complication was also reflected in single-cell RNA-seq experiments as a study performed on splenic conventional T cells (T_{conv} cells; defined as CD4+ TCRβ+Foxp3+) and Treg cells (defined as CD4+ TCRβ+Foxp3+) from Foxp3^{GFP} reporter mice revealed, based on unsupervised clustering in a tSNE plot, a cluster of cells enriched in T_{FH}/T_{FR} cell-associated genes. Interestingly, the cluster is composed of both T_{conv} and Treg cells, implying that potential T_{FH} cells and T_{FR} cells, while having markedly opposite functions, might possess related transcriptional profiles that are overlapping despite the clear distinction observed with the Foxp3 reporter expression (156). The study, however, only identified cells based on T_{FH}/T_{FR} signature genes. The inclusion of the cells
based on CXCR5 and PD-1/ICOS expression at the protein level or with Bcl6 reporter is still needed to confirm that the cells being analyzed are indeed $T_{FH}/T_{FR}$ cells.

**Germinal Centers**

Antibody responses are among the hallmarks of the immune system. Germinal centers (GCs) are micro-anatomical structures that emerge in secondary lymphoid organs upon exposure to antigen through infection or immunization (157, 158). These structures are the sites where affinity maturation, plasma cell generation and memory B cell differentiation occur (159-161). Formation of GCs upon antigen exposure begins at B cell follicles in secondary lymphoid organs, which are interspersed within a network of stromal-derived follicular dendritic cells (FDCs) (162). In B cell primary follicles, FDCs provide organizational support by helping B cells to aggregate into compact, well-defined clusters (163). Once GCs are formed, however, FDCs can serve as a major reservoir for antigens as FDCs were shown to acquire and cycle complement-coated immune complexes between FDC surface and nondegradative endosomal compartments, resulting in protection of antigens from damages and prolonged retention of those antigens while maintaining their accessibility for B cells (164). In addition, FDCs can also promote B cell activation *in vitro* (165-167) and TLR4 signaling in FDCs supports GC B cell survival, which is pivotal for GC onset and affinity maturation *in vivo* (168). FDCs also produce B-cell activating factor (BAFF) (169), which rescues GC B cell from apoptosis (170).

Initiation of the GC reaction begins when naïve B cells acquire antigens (171-173). Antigen-primed B cells then migrate to the border of T cell and B cell zones or the interfollicular regions where they proliferate and interact with cognate T cells to become fully activated (174-176). Some of the activated B cells with relatively high affinity enter B cell follicles (177, 178)
as these B cells outcompete others for T cell signals (179). T/B border zones and interfollicular regions are also the location where GC B cell and T_{FH} cell phenotypes are initiated (180, 181).

Establishment of GCs is marked by compartmentalized division into dark zones (DZs) and light zones (LZs). DZs consist of compactly-clustered proliferative “centroblast” GC B cells expressing the chemokine receptor CXCR4 (182-185) that responds to chemokine CXCL12 made by Cxcl12-expressing reticular cells (186, 187). DZ GC B cells express a high level of AID and are prone to somatic hypermutation (SHM) (188), thus making them to be a pool of clonal variants to be selected (184, 185). LZs, on the other hand, are less compact zones, consisting of “centrocytes” GC B cells, FDCs, T_{FH} cells and T_{FR} cells. LZ GC B cells possess more activated phenotypes, expressing higher activation markers like CD86 and CD83, as well as gene signatures associated with CD40 and BCR pathways (184, 189). 10%-30% of B cells that arrive to the LZ are selected to re-enter the DZ while the remaining cells either die by apoptosis or exit the GCs (184). DZ/LZ interzonal migration is dependent on a pre-programmed intrinsic number of cell divisions in B cells (190). Ubiquin-mediated fluctuations in MHC-II-antigen complexes were shown to promote MHC-II turnover, thus facilitating the replacement of old complexes and allowing GC B cells to re-enter LZ for additional rounds of antigen acquisition and presentation (191). The uses of anti-DEC205 antibodies conjugated with antigens, which allow antigen uptake without activating BCRs, demonstrated that T_{FH} cell help, rather than BCR signaling, is the major factor that drives selection of high-affinity GC B clones through cyclic interzonal migration as the cells undergo SHM and clonal selection in a repeating fashion (184, 190). However, it was also shown that although BCR crosslinking is not required for GC B cell selection and plasmablast differentiation, it could synergize with T_{FH} cell help when such help is sub-saturated (192). As mentioned in a previous section, T_{FH} cells can provide help in multiple
ways, including through the cytokines IL-21, IL-4, CD40LG (193), and BAFF (194) and by engagement of feed-forward ICOS-ICOSL interactions (195). Without T<sub>FH</sub> cell help, GC B cells, as a default fate, are dying by apoptosis as they express high levels of Fas and low levels of Bcl-2 (196-199).

Single B cell transfer experiments revealed that surviving GC B cells can take 3 fate decisions: 1) recycling within GCs 2) exit GCs as plasma cells or 3) exit GCs as memory B cells, depending BCR affinity. GC B cells with high and low affinity exit GCs as plasma cells and memory B cells respectively, while GC B cells with intermediate affinity remain recycling in GCs. About half of individual naïve B cell clones only undergo one fate decision, highlighting the importance of BCR affinity in such fate determination. However, the fate decision is not determined by affinity alone, as a small portion (5%-10%) of B cell clones gave rise to all three fates, probably due to the extent of contextual help independent of BCR affinity (200). At the molecular level, c-Myc was shown to be important for GC B cell proliferation. c-Myc is briefly but strongly induced in about 10%-30% of LZ B cells that undergo positive selection, as enforced T<sub>FH</sub> cell help strongly up-regulates c-Myc in B cells while c-Myc expression is shut down in DZs. A small portion (10%) of c-Myc<sup>+</sup> LZ GC B cells also express IRF4 (189, 201). These LZ GC B cells were shown to have higher BCR affinity than c-Myc<sup>−</sup>IRF4<sup>+</sup> B cells, resulting in their favored GC exit as plasma cells. The Myc<sup>+</sup>IRF4<sup>+</sup> B cells also require more T<sub>FH</sub> cell help in a CD40-dependent fashion and they were demonstrated to be precursors that further develop into Blimp-1<sup>+</sup> plasmablasts (202, 203). Blocking antigen access, but not T<sub>FH</sub> cell-derived CD40L, prevents initiation of plasma cell differentiation in the GCs. On the other hand, T<sub>FH</sub> cells are essential for completing the differentiation process and drive migration of maturing plasma cells through the dark zone and out of the GCs (202), a process driven by T<sub>FH</sub> cell-derived IL-21
(204-206) and B cell induction of Prdm1 (encoding Blimp-1) (207, 208), which serve as extrinsic and intrinsic drivers of plasma cell differentiation respectively.

Memory B cell generation, however, is initiated in low affinity LZ GC B cells at the early GC stage (209, 210) as limited help by T_{FH} cells results in induction of transcription factor Bach2, which mediates the generation of memory B cells (209). In mice that are deficient in the chemokine receptor CXCR4, in which B cells cannot enter the DZ and T_{FH} cell help was misdirected, the memory B cell compartment was shown to be enhanced (186). In addition, IL-21R deficiency in B cells using mixed bone-marrow chimera experiments led to an increase in memory B cell generation (48, 49). Exposure to differential help by T_{FH} cells, therefore, influences how GC B cells differentiate into different fates. Interestingly, it was recently shown that T_{FH} cell-derived IL-9 favors memory B cell differentiation (211). At a glance, such findings appear to contradict the earlier model in which T_{FH} cell help is supposed to disfavor the memory B cell differentiation pathway. However, it was suggested that comparative abundance — 20%-40% of T_{FH} cells being IL-21^+ (212) versus only about 2% of T_{FH} cells being IL-9^+ — might affect the relative GC output of plasma cells versus memory B cells. Whether IL-21^+ T_{FH} cells and IL-9^+ T_{FH} cells harbor different TCRs with differential affinity remains to be investigated. Another potential mechanism, given that IL-9 also enhances the suppressive function of Treg cells (213), might be due to IL-9 from T_{FH} cells acting on T_{FR} cells, which then limit T_{FH} cell help, resulting in the bias toward memory B cell generation.

**Biology of Fgl2**

As fibrinogen-like protein 2 (Fgl2) is a major molecule that will be investigated and discussed in subsequent chapters, its detailed biology is reviewed here.
Fgl2 Structure and Expression

The Fgl2 gene was first cloned from a cytotoxic T lymphocyte cDNA library as a gene with 36% homology to fibrinogen β and γ chains (214), and thus identified as a member of the fibrinogen superfamily. The gene is located on chromosome 7 in humans and chromosome 5 in mice, with 80% homology between the two species (215). The full-length Fgl2 protein has 439 amino acids in humans and 432 amino acids in mice, with a predicted molecular weight of 50 kDa (215, 216). However, two bands of 65 kDa and 70 kDa were seen under reducing SDS-PAGE conditions, and enzymatic deglycosylation resulted in a 55 kDa band, indicating that the protein is glycosylated (216, 217). Under non-reducing SDS-PAGE conditions, Fgl2 migrates with a molecular weight of approximately 260 kDa as it forms an oligomer consisting of 4 monomers (215, 217, 218).

The N terminal of the Fgl2 protein harbors conserved cysteine residues (219), which enable its oligomerization via disulfide bonds in coiled-coil structure (215). Four of those cysteine residues (Cys^{94}, Cys^{97}, Cys^{184} and Cys^{187}) in the coiled-coil domain, linearly arranged as two pairs in a Cys-X-X-Cys motif, were shown to be important for Fgl2 inter-chain quaternary structure as the of loss of any two of these four residues led to partial expression of monomeric Fgl2 while the loss of all of them led to a predominance of its monomeric form. The C terminal of Fgl2 protein contains a fibrinogen-related domain (FRED) with 90% homology between humans and mice (220). This domain accounts for not only its binding to its receptors but also its coagulation activity and immunoregulatory effects (217). Upon assembly, Fgl2 forms an asymmetric bouquet-shaped structure, similar to many pattern-recognition molecules in the lectin pathway of innate immunity with the C terminal at the bouquet head (shown in Figure 1.5) (217).
Fgl2 protein has been shown to exist in 2 forms: type II membrane-bound and soluble (216, 221, 222). The membrane-bound form was shown to possess prothrombinase activity in certain contexts such as during murine hepatitis virus-3 (MHV-3) pathogenesis (223). However, the expression of membrane bound Fgl2 is limited to endothelial cells, cardiomyocytes and macrophages, but not CD4 or CD8 T cells (221, 223). The secreted form of Fgl2, on the other hand, only contains the C-terminal FRED domain (217). Since a phospholipid membrane containing phosphatidyl-L-serine, Ser\textsuperscript{89}, is required for Fgl2 prothrombinase activity, secreted Fgl2, which lacks the domain harboring that serine, does not possess activity (222).

\textbf{Figure 1.5:} SWISS-MODEL prediction of mouse full-length, membrane-bound Fgl2 structure. A) A ribbon format showing a tertiary structure of monomeric Fgl2 with α helices shown in red, β shown in blue and cysteine residues shown as green spheres. The hydrophobic region in the N terminal was predicted to be either transmembrane domain or signal peptide. B) Tertiary structure of tetrameric Fgl2 shown in both side and top views based on oligomerization and deglycosylation analysis. Disulfide forming cysteine residues shown in red and carbohydrate
moieties shown in blue. The secreted form of Fgl2, only contains the C-terminal FRED domain. The figure is reproduced from Liu et al., 2013 with copyright permission acquired.

Fgl2 expression has been reported in multiple cell types and tissues, including Treg cells, cardiomyocytes, reticuloendothelial cells, intestinal lamina propria myeloid cells, epididymis and trophectoderm (215, 221, 224-227). However, the constitutive expression of Fgl2 mRNA was shown to be low except for its expression in Treg cells (216, 220, 228). Furthermore, Fgl2 expression can be induced. In macrophages, IFN-γ, but not LPS, TNF-α, IL-1 and IFN-α, can up-regulate the expression of Fgl2 mRNA (229). In endothelial cells, however, Fgl2 was shown to be induced by TNF-α but not IFN-γ (230). It was shown that a 119 bp region upstream of the Fgl2 transcription start site contains multiple cis-regulatory elements including Oct-1, Sp1/Sp3 and Ets-1/STAT1 binding sites (230, 231). This region is required for induced Fgl2 expression in macrophages and endothelial cells by IFN-γ and TNF-α respectively (230). In Treg cells, gene expression analysis revealed Fgl2 as a gene regulated by IL-2 (232). In addition, a functionally distinct subset of Treg cells expressing the co-inhibitory molecule TIGIT was shown to express a high level of Fgl2, and TIGIT ligation with an agonistic anti-TIGIT antibody resulted in its further up-regulation mediated by a transcription factor C/EBPα (117). Other works also demonstrated further evidence on Fgl2 regulation by viral proteins. For example, hepatitis B core protein (HBc) and hepatitis B virus X protein (HBx) enhance transcription of Fgl2 through c-Ets-2, which is dependent on MAPK signaling pathways based on Luciferase reporter experiments, while up-regulation of Fgl2 was associated with increased pJNK in activated alveolar macrophages in chronic obstructive pulmonary disease patients (233). It was suggested that these MAPK signaling pathways might also underlie Fgl2-driven feedforward regulation as
knockdown of Fgl2 in human hepatocellular carcinoma cells resulted in decreased pERK and pJNK while Fgl2 overexpression yielded the opposite effects (234). The pathways were also implicated in murine hepatitis virus 3 (MHV-3) infection as Fgl2 expression was shown to be dependent on p38 (235). Taken together, induction of Fgl2 can be achieved by viral proteins and danger signals such as inflammatory cytokines in a variety of cell types, suggesting the potential involvement of Fgl2 in diverse contexts.

Fgl2 receptors

Thus far, two receptors for Fgl2 in mice have been identified: FcγRIIb and FcγRIII (230, 236). These two receptors are low-affinity Fc receptors for IgG antibodies, either in the forms of immune complexes or free antibodies (237). The two low-affinity receptors, however, were considered to possess opposing signaling effects as FcγRIII contains activating ITAM motifs while FcγRIIb contains inhibitory ITIM-motifs (238). Previous works demonstrated that FcγRIIb is expressed in all myeloid and lymphoid cells, especially B cells, whereas FcγRIII is expressed in myeloid cells. Expression of the two receptors on T cells, however, has been shown to be limited (237) but whether their expression is present on specific T cell subsets and/or T cell activation states remains to be further investigated.

Recombinant tetrameric Fgl2 protein is capable of binding to B cell lines, macrophages and DCs, and this binding is antagonized upon treatments with Fc block, which interferes with the binding to both receptors. Surface plasmon resonance also confirmed the specificity of Fgl2 binding to FcγRIIb and FcγRIII but not FcγRI, VCAM-1 or ICAM-1 (236). Interestingly, the affinity of FcγRIIb binding to Fgl2 appears to be higher than for human IgG2, as the binding affinity of Fgl2 to FcγRIIb/FcγRIII in murine RAW264.7 cell lines is 20 nM (236) while that of
Fcγ2b binding to human IgG2a is approximately 0.6 μM (239). More appropriate, detailed comparisons, however, are required as there could be discrepancies in properties between murine and human proteins. Moreover, human FcγRIII protein, unlike that in mice, has two forms: FcγRIIIa and FcγRIIIb (237), and the relevance of these forms with respect to Fgl2 binding and functions have never been explored.

As Fcγ receptors exist as activating and inhibitory proteins, cellular activation can be regulated by the balance of these receptors in response to inflammation and cytokine environments (240). Signaling cascades downstream of FcγRIIb have been relatively well characterized. FcγRIIb is a single chain receptor with its cytoplasmic tail containing an inhibitory ITIM motif. Upon engagement with immune complexes, phosphorylated ITIMs in FcγRIIb recruit phosphatases like SHPs, which in turn antagonizes activating ITAM-mediated signals from other receptors like activating Fc receptors and/or BCRs in B cells. Such antagonism limits ITAM-dependent mobilization of calcium ions and the downstream NFAT pathway, as well as MAPK pathways required for ITAM-mediated proliferation. FcγRIIb can also homo-aggregate, resulting in ITIM-independent apoptosis, which is a mechanism to eliminate autoreactive B cells and to prevent the accumulation of plasma cells (240-242).

FcγRIIb-deficient mice on the mixed C57BL/6 and 129/Sv background develop spontaneous accumulation of autoantibodies and glomerular disease with premature mortality, identifying it as a lupus susceptibility gene (243). In addition, immunization of a nonpermissive, H-2b strain of mice like C57BL/6 with bovine collagen type II or IV, resulted in loss of tolerance with the development of anti-mouse collagen auto-antibodies and the subsequent development of arthritis and a Goodpasture disease-like phenotype respectively (244, 245) FcγRIIb-deficient mice derived on the C57BL/6 background had enhanced susceptibility to collagen-induced
arthritis (246) although their spontaneous lupus-like phenotypes, including proteinuria and premature mortality, were attenuated compared to those found in the mixed C57BL/6 and 129/Sv background, implying the presence of additional susceptibility factors contributed by 129/Sv genes (247). Other studies also demonstrated that autoimmunity-susceptible mouse strains, such as NZW, BXSB and NOD, displayed defective regulation of FcγRIIb expression with polymorphisms in the promoter region of the gene, leading to a dysregulation of B cell activation (248). Replacement of the FcγRIIb gene in C57BL/6 mice with the autoimmunity-associated alleles was shown to result in multiple autoimmune phenotypes, including the development of more severe collagen-induced arthritis (249). Defects in FcγRIIb expression or function were described in human SLE populations in which it had been observed that 0.50% of lupus patients fail to upregulate FcγRIIb upon B cell activation (250). Loss-of-function polymorphisms affecting FcγRIIb expression and signaling functions have also been identified in different cohorts of SLE populations (251-254). Unlike FcγRIIb, FcγRIII, however, is much less studied. FcγRIII-deficient mice were shown to have defects in NK cell-mediated antibody-dependent cytotoxicity, phagocytosis of IgG1-coated particles by macrophages, and IgG-mediated mast cell degranulation. The mice were also resistant to IgG-dependent passive cutaneous anaphylaxis, and exhibited an impaired immune complex-mediated type-III hypersensitivity Arthus reaction (255). A polymorphism in FcγRIII was also shown to be associated with SLE although the underlying biology of this polymorphism has not been characterized (256)

**Fgl2 Functions: Prothombinase Activity and Immunomodulatory Effects**

Fgl2, in its membrane-bound form, was first shown to be a prothombinase expressed by macrophages and endothelial cells during MHV-3 infection and was associated with fatal
fulminant hepatic failure that was characterized by fibrin deposition and hepatocellular necrosis (228). Fgl2 prothrombinase activity is implicated in a wide array of diseases associated with pathological deposition of fibrin, including acute fulminant hepatitis, SARS-like infection, hepatitis B infection, rheumatoid arthritis and xenotransplantation (224, 226, 257-259). Treatment of MHV-3-infected mice with anti-Fgl2 antibodies attenuated fibrin deposition and disease severity (260) while Fgl2-deficient mice were shown to be free of fibrin deposition and less susceptible to MHV-3 infection and collagen-induced arthritis models (224, 259). Targeted deletion of Fgl2 in mouse to rat cardiac xenografts, moreover, led to reduced serum fibrin, antibody and fibrin deposition, corresponding to lower acute vascular rejection (261, 262). Interestingly, while Fgl2 appears to be important in fibrin deposition in the contexts described above, it was shown to be indispensable in other contexts associated with fibrin deposition, including infection with *Toxoplasma gondii, Yersinia enterocolitica, Listeria monocytogenes* and *Mycobacterium tuberculosis*, as well as BALB/c to C57BL/6 allotransplantation (262). Such results suggest that resolution of fibrin deposition mediated by Fgl2 is specific to only particular infectious pathogens and/or inflammatory conditions.

Fgl2, in its soluble form, has been shown to be as an immunoregulatory molecule, a feature attributed to its FRED domain. Its effects have been shown in several immune cell types, including B cells, dendritic cells and effector T cells. Soluble recombinant Fgl2 was shown to suppress T cell proliferation *in vitro* without affecting apoptosis (216) and to skew the cytokine profiles from those of Th1 cells toward those of Th2 cells in allogenic mixed-lymphocyte reaction experiments (236). In B cells, however, soluble Fgl2 was shown to induce apoptosis in the A20 B cell line while the A20IIA1.6 B cell line, which lacks FcyRIIb, was completely resistant to Fgl2 cytotoxicity, suggesting the requirement of the receptor (236). The addition of
soluble Fgl2 to LPS-stimulated bone marrow derived dendritic cells (BMDCs) resulted in decreased DC maturation based on reduced expression of MHC-II and CD80, as well as a defective capacity to stimulate T cells. These phenotypes were associated with decreased nuclear translocation of the p65 subunit of NF-κB and they were completely abolished when BMDCs were Fcgr2b-deficient. Effects on DC expression of CD86 and CD40 were inconsistent in different studies (216, 236). As mentioned earlier, Fgl2 was identified as a key effector molecule in TIGIT+ Treg cells and TIGIT ligation resulted in robust production of Fgl2 and IL-10. These TIGIT+ Treg cells were able to selectively suppress Th1 cells and Th17 cells but not Th2 cells. The suppression of Th1 and Th17 cells due to Treg cells-derived Fgl2 was demonstrated based on T cells in vitro suppression cultures and adoptive transfer experiments into RAG1-deficient mice (117). Immunization with lectins and alloantigens in Fgl2-deficient mice, moreover, resulted in increased in T cell proliferation, enhanced IFN-γ production by T cells, up-regulation of CD80 and MHCII in DCs and increased yet dysfunctional Treg cells. These phenotypes in the cohort corresponded with spontaneous development of glomerulonephritis, but splenic architecture and germinal centers appeared to be normal (227). Infection of Fgl2-deficient mice with both acute and chronic LCMV strains led to enhanced DC maturation, CD4 T cell production of IFN-γ and increased virus-specific CD8 T cells and neutralizing antibodies, consistent with better clearance of the viruses over time (263). Fgl2 has also been implicated in cancer as it has been shown to be expressed in glioblastoma, colorectal carcinoma and hepatocellular carcinoma cells (264-266). In glioblastoma, malignancy was shown to correspond with high copy number mutations of the Fgl2 gene and its mRNA level in the tumor was inversely correlated with the disease outcome. Overexpression of Fgl2, in addition, was shown to enhance tumor growth in a glioblastoma mouse model while anti-Fgl2 antibody treatment led to
slower tumor growth with increased median survival (264). In tolerance transplantation, rapamycin-induced tolerance in a murine heart transplant model was shown to be dependent on Treg cells and their expression of Fgl2, as treatment with Treg-depleting anti-CD25 antibodies or anti-Fgl2 neutralizing antibodies led to allograft rejection (267). Treatment with recombinant Fgl2 was also shown to prevent rejection of mismatched cardiac allografts, but the rejection resumed once the treatment was stopped. Consistent with such findings, Fgl2 transgenic mice were found to spontaneously accept 50% of allografts without additional immunosuppression (268). Collectively, these findings reveal Fgl2 as an immunosuppressive factor with pleiotropic effects on a wide variety of immune responses in diverse physiological and pathological contexts.

Taken together, we reviewed the concepts of lineage commitment, plasticity and functional heterogeneity in CD4 T cell subset differentiation. We then apply our current understanding of CD4 T cell subsets to exhibit outstanding questions regarding follicular helper T cells and follicular regulatory T cells with respect to their shared features with other known CD4 T cell subsets. In addition, we reviewed the biology of germinal centers with respect to their initiation, dynamic reactions and effector outcomes. Finally, we provided detailed description on Fgl2, which is a major molecule to be investigated and discussed in subsequent chapters.
Chapter 2

TFR cell-derived Fibrinogen-like Protein 2 Promotes Expression of Coinhibitory Molecules on T<sub>FH</sub> Cells and Regulates Antibody Responses
Abstract

Follicular regulator T (T\textsubscript{FR}) cells limit antibody responses but the underlying mechanisms remain largely unknown. Here, we identify fibrinogen-like protein 2 (Fgl2) as a soluble T\textsubscript{FR} cell effector molecule through single-cell gene expression profiling. Highly expressed by T\textsubscript{FR} cells, Fgl2 directly binds to B cells, especially light-zone germinal center B cells, as well as to T\textsubscript{FH} cells and directly regulates B cells and T\textsubscript{FH} cells in a context-dependent and type 2 antibody isotype-specific manner. In T\textsubscript{FH} cells, Fgl2 induces the expression of Prdm1 and a panel of checkpoint molecules including PD-1, TIM-3, LAG-3 and TIGIT, resulting in T\textsubscript{FH} cell dysfunction. Mice deficient in Fgl2 had dysregulated antibody responses at steady state and upon immunization. In addition, loss of Fgl2 results in expansion of autoreactive B cells upon immunization. Consistent with this observation, aged Fgl2\textsuperscript{-/-} mice spontaneously developed autoimmunity associated with elevated autoantibodies. Thus, Fgl2 is a T\textsubscript{FR} cell effector molecule that regulates humoral immunity and limits systemic autoimmunity.


Introduction

B cell-mediated antibody production is a major component of adaptive immunity. Multiple processes that contribute to optimal antibody responses depend on a histologically specialized site called the germinal center (GC) located in B cell zones of secondary lymphoid organs. Such processes include B cell affinity maturation, class switch recombination, plasma cell differentiation and memory B cell generation (35, 269). GC formation and maintenance, and antibody class switching depend on help from specialized CD4+ follicular helper T (T_{FH}) cells (270), which were first described as CD4+ T cells expressing a high level of the chemokine receptor CXCR5 that drives T_{FH} migration to B cell follicles in response to the chemokine CXCL13 (36-38). T_{FH} cells express the transcription factor Bcl-6, a master regulator that mediates a unique T_{FH} transcriptional program, while the transcription factor Blimp-1 (encoded by the gene Prdm1) antagonizes Bcl6 and inhibits T_{FH} differentiation and help (39-41). In addition to the expression of Bcl6, T_{FH} cells express c-Maf, which induces IL-21 production (86) and Ascl2, which is critical for the expression of CXCR5 and trafficking of T_{FH} cells into the GCs (271). T_{FH} cells also express high levels of the co-stimulatory molecule ICOS and the co-inhibitory molecule PD-1. These cells provide help to B cells through co-stimulatory molecules and cytokines, including CD40L, IL-21 and IL-4 (36, 86, 136, 138, 204, 272-275).

Recently, a CXCR5\textsuperscript{+}Foxp3\textsuperscript{+} CD4 T cell population was shown to mediate inhibitory effects on germinal center reactions and antibody responses, and thus the cells were defined as T follicular regulatory T (T_{FR}) cells (146-148). Moreover, the cells have been shown to limit autoimmune responses in animal models of influenza infection and Sjogren’s disease (149, 150). Unlike T_{FH} cells, however, Bcl6 and Blimp-1 are co-expressed in T_{FR} cells. Blimp-1 has been previously shown to influence Treg function by inducing an effector regulatory phenotype (151).
It is expressed by Treg at mucosal sites and by a small subset of splenic Treg cells that produce IL-10 in a Blimp-1-dependent manner (276). Similar to these Treg cells, T弗 cells share high expression of IL-10, GITR and ICOS. Thus, T弗 cells were suggested to be the follicular counterparts of the Blimp-1\(^+\) IL-10\(^+\) effector Treg cells found at mucosal surfaces (276, 277) and in addition, because of their presence in GCs, to regulate T cell dependent antibody production by B cells. Recent studies suggested that T弗 cells are able to durably alter multiple metabolic pathways in B cells through epigenetic changes in the B cells, resulting in their diminished antibody production (278). Expression of the co-inhibitory receptor CTLA-4 on T弗 cells was demonstrated to be important for T弗 cell effector functions as the loss of CTLA-4 on T弗 cells led to their reduced capacity to suppress antibody responses (152, 153). However, the effector mechanisms utilized by T弗 cells to suppress B cells, DCs or other helper T cells, have not been fully investigated.

We performed population and single-cell RNA-seq assays of CD4\(^+\) T cells and computationally analyzed their transcriptome to identify soluble molecules that are differentially expressed in T弗/effector Treg cells in comparison to T弗 cells or conventional Treg cells. We identified fibrinogen-like protein 2 (Fgl2) as a T弗 cell effector molecule and a direct regulator of antibody responses. The molecule binds to B cells, particularly LZ GC B cells, and T弗 cells, and thereby directly modulates class-switch recombination in B cells and cytokine production from T弗 cells. The inhibitory functions of Fgl2 were partly due to its effects on T弗 cells as it induces expression of Prdm1 and a panel of “co-inhibitory” checkpoint molecules. Fgl2-deficient mice have dysregulated homeostatic and immunization-induced antigen-specific antibody responses. Fgl2-deficient T弗 cells failed to suppress IgG1 production in vitro and in vivo. Moreover, aged Fgl2-deficient mice showed signs of autoimmunity with elevated autoantibody level. Together
these findings demonstrate that Fgl2, a TFR effector molecule, regulates Tfh cell and B cell functions and loss of Fgl2 results in dysregulated autoantibody responses and development of systemic autoimmunity.

**Materials and Methods**

**Mice.** Wild-type C57BL/6J, FcγRIIB−/−, FcγRIII−/− and Prdm1Tfl mice were purchased from Jackson Laboratories. FoxP3RES-GFP and Fgl2−/− mice on the C57BL/6 background have been published previously (22, 227). Fgl2−/− with FoxP3RES-GFP reporter mice were bred in the lab. CD28+ mice on the C57BL/6 background were from Arlene Sharpe lab. All mice were between 6 and 8 weeks of age at the time of experiments unless specific ages were mentioned in particular experiments. All mice used in each experiment were age-matched and gender-matched. The experiments were conducted in accordance with animal protocols approved by the Harvard Medical Area Standing Committee on Animals or BWH IACUC.

**Immunizations.** Mice were subcutaneously immunized with 100 μg NP-OVA (Biosearch Technologies) emulsified in Complete Freund's Adjuvant (BD) in the mouse flanks as previously described (279). For immunization to induce autoreactive B cells, additional heat killed dried *Mycobacterium tuberculosis* was added to CFA to the final concentration of 4 mg/mL. Mice were sacrificed later and inguinal lymph nodes were harvested.

**Antibodies.** The following antibodies were used for surface staining: anti-CD4 (RM4-5), anti-CD19 (6D5), anti-CXCR5 biotin (2G8), anti-PD-1 (RMP1-30), anti-FAS (15A7), GL7 (GL-7), anti-IgM (RMM-1), anti-IgD (11-26c.2a), anti-B220 (RA3-6B2), anti-CD38 (90), anti-CD138 (281-2), anti-CXCR4 (L276F12), anti-CD86 (GL-1), anti-His tag (AD1.1.10) and anti-TIM-3
(5D12). Secondary staining for biotinylated primary antibody was done using streptavidin (BioLegend). For intracellular staining samples were fixed with Fixation/Perm solution kit (BD) for intracellular Ig or FoxP3 Fix/Perm buffer set (eBioscience) for staining transcription factors according to manufacturer’s instruction. Samples were then intracellularly stained with anti-IgG1 (RMG1-1), anti-IgG2b (RMG2b-1), anti-IgE (RME-1), anti-IgA (goat anti-mouse polyclonal, Southern Biotech), anti-FoxP3 (FJK-16S) and anti-Bcl6 (K112-91). For Fc block experiments, 10 ng/mL purified anti-mouse CD16/32 antibody (BioLegend) was used. For anti-IL10 blocking experiments, 500 ng/mL of purified rat anti-mouse IL-10 (BD) was used.

**Sorting.** Single cell suspensions were diluted in PBS supplemented with 3% FBS with 2 mM EDTA. CD4⁺ cells were enriched by magnetic positive selection (Miltenyi Biotec). CD4⁺ enriched cells were then stained and sorted as follows: TFH (CD4⁺ CD19⁺ICOS⁺CXCR5⁺FoxP3⁺), TFR (CD4⁺ CD19⁺ICOS⁺CXCR5⁺FoxP3⁺). B cells were isolated from flow-through from CD4⁺ selection and sorted as CD19⁺CD4⁻. Single sorting was used in all of the experiments, except for gene expression profiling ones, in which double sorting was used.

**Droplet-based single-cell RNA-Seq.** Inguinal lymph nodes from wild-type mice immunized with NP-OVA/CFA (s.c.) for 7 days were isolated and CD4⁺ cells by magnetic positive selection (Miltenyi Biotec). Cells were then sorted based on CD19⁺CD4⁺CXCR5⁺PD-1⁺ with permissive thresholds for CXCR5 and PD-1 gating. Cells were then subjected to droplet-based single-cell RNA-seq using the Chromium Single Cell Gene Expression Solution platform (10x Genomics).

**Population RNA-Seq.** Samples were sorted as described above. RNAseq library preparations were performed. Briefly, RNA was isolated using RNeasy Mini Kit (Qiagen). The cDNA libraries were prepared based on modified SMART-seq2 protocol as previously described with 8...
amplification cycles(111, 280). The library quality was confirmed using BioAnalyzer high sensitivity DNA chip (Agilent Technologies). RNA Sequencing reactions were sequenced on an Illumina HiSeq 2000 or Illumina NextSeq sequencer (Illumina) according to manufacturer’s instructions, sequencing 50bp reads.

**In vitro culture assays.** In vitro culture assays were performed as previously described(281). Briefly, 5x10^4 B cells and/or 3x10^4 T_{FH} cells and/or 1.5x10^4 T_{FR} cells were plated in 96-well plates along with 25 ng LPS or 2ug/ml anti-CD3 and 5ug/ml anti-IgM (Jackson Immunoresearch). Polarizing cytokines for T cells and B cells class-switch recombination are described in specific experiments. Cultures were harvested after 3-6 days as specified in specific experiments. Recombinant tetrameric mouse Fgl2-His (R&D Systems) was added into the culture as described. For analysis, B cells were gated as CD19^+CD4^- cells while specific isotype staining was done by intracellular staining. T_{FH} cells were gated as CD4^+CD19^-FoxP3^- cells, and T_{FR} cells were gated as CD4^+ CD19^-FoxP3^+ cells.

**ELISA.** ELISA measurements of IgG from culture supernatants and sera were performed as described previously(279, 282). For autoantibody ELISA, mouse anti-nuclear antigens (ANA/ENA) Ig's (total (A+G+M)), anti-dsDNA Ig's (Total A+G+M) and anti-dsDNA IgG1-specific ELISA kits were used according to the manufacturer’s instruction.

**Bead-based immunoassays.** Soluble mouse Ig isotype measurements were performed using mouse immunoglobulin isotyping kit (BD) while soluble cytokine measurements were performed using LEGENDPlex kit (BioLegend) according to manufacturers’ instructions. The mouse immunoglobulin isotyping kit provides no standard curve assessment so the results should be considered more as qualitative. The LEGENDPlex kit, however, provides standard curve assessment.
**Autoantigen microarray.** Lupus autoantigen microarrays were constructed and developed as described (283-285). Briefly, 33 were spotted onto Epoxy slides (Arrayit Corporation, Sunnyvale, CA, USA). The microarrays were blocked for 1h at 37°C with 1% bovine serum albumin and incubated for 2 hr at 37°C with a 1:100 dilution of the test serum in blocking buffer. The arrays were then washed and incubated for 45 minutes at 37°C with a 1:500 dilution of a goat anti-mouse IgG detection antibody conjugated to Cy3 and anti-mouse IgM detection antibody conjugated to Cy5 (Jackson ImmunoResearch, West Grove, PA). The arrays were scanned with a ScanArray 4000X scanner (GSI Luminomics, Billerica, Massachusetts, USA).

**ELISPOT.** Ethanol-activated MultiScreenHTS IP filer plates (EMDMillipore) were coated with either 100 μL of filtered (with 0.45 μM filters) Salmon Sperm DNA (5 μg/mL g/mL; ThermoFisher) or NP-OVA (10 μg/mL, BiosearchTech) overnight at 4°C. The plates were then washed with PBS and blocked for 2 hours with blocking buffer (5% FCS, 3% BSA in PBS) before they were re-washed and let dry. 50 μL of splenocytes in clone media (started with 5 x 105 cells per well with 1/3 serial dilutions) were plated and 50 μL of αCD40 (10 μg/mL) was added per well (final concentration of 5 μg/mL). The cells were incubated at 37°C for 24 hours. After that, cells and unbound cytokines were washed by incubating with PBS Tween-20 buffer for 10 minutes and then thoroughly re-washed. 50 μL of biotin-conjugated goat anti-mouse Ig (1:350 dilution in clone media, SouthernBioTech) was then added and incubated overnight at 37°C for primary staining. After rewashing, secondary staining was done for 1 hour using 50 μL of streptavidin-ALP (1:1000 dilution in 1% BSA in PBS, Mabtech). The plates were then washed and developed using 50 μL BCIP/NBT-plus substrate (Mabtech). When spots are clearly visible under a dissecting microscope, stop the development by discarding the substrate and rinse plates with tap water thoroughly. Spots were counted manually with a dissecting microscope.
**Nanostring.** nCounter platform (NanoString Technologies) was used according to the manufacturer’s instruction. A codeset of T cells associated genes and 4 additional house-keeping genes were custom-made.

**Histology and Immunohistochemistry.** Spleens, lymph nodes, lungs, kidneys, livers, guts and patches of skin were harvested and fixed in 10% formalin, paraaffin-embedded, and sectioned. Representative sections were stained with hematoxylin & eosin. For immunohistochemistry, avidin-biotin immunohistochemical staining was performed on the sections with rabbit anti-mouse CD3 (Abcam, Cambridge, UK) and rat anti-mouse CD45R (B220; BD Biosciences) using reagents from Vector Laboratories (Burlingame, CA).

**Statistical analysis for non-RNA-Seq data.** Statistical analysis was performed using Prism 7 (GraphPad). Differences between two groups were compared using two-tailed unpaired T tests and differences between two groups in multiple comparisons were done using one-way ANOVA (n.s; * p <0.05, ** p<0.01 and *** p<0.001). All figures show the means ± SD.

**Data analysis for scRNA-Seq data.** 10x sequencing outputs were processed with Cell Ranger (10x Genomics), and loaded into a Seurat object(286), which was used to scale the data, regress out unwanted axes of variation (number of UMIs and ratio of mitochondrial UMIs per library), and cluster the cells with the SLM algorithm. Default parameter values were used unless specified otherwise. nTreg and TFH signatures are manually curated. We computed an unsupervised signature based on TFH vs. TR cells by calling differentially expressed (DE) genes in population RNA libraries described earlier. Libraries were aligned with Tophat2(287), reads per transcript were counted with featureCounts(288) and DE genes were then called with DESeq2(289) using the thresholds FDR <= 10% and |B| >= 10% where B is the moderated B-statistic. Given a gene expression matrix $A_{m\times n}$ for m genes over n cells, a signature is a column
vector $v_m$ of length $m$ with values of 1, -1, and 0 corresponding to upregulated, downregulated, and non-DE genes, respectively. The signature values for the cells are the ranked-transformed values of $A^T v$.

**Results**

**Fgl2 is a distinguishing marker for $T_{FR}$ cells**

To assess potential effector molecules downstream of $T_{FR}$ cells, we performed a high-throughput 10x single-cell RNA-Seq assay on CD19$^+$CD4$^+$CXCR5$^+$PD-1$^+$ T cells from draining lymph nodes of wild-type mice immunized with NP-OVA/CFA for 7 days. We used permissive thresholds for CXCR5 and PD-1 gating, which included some cells lacking both markers, in order to gain a complete statistical representation of $T_{FR}$ cells states (290). More restrictive gating would preferentially exclude $T_{FR}$ states where CXCR5 or PD-1 are present but are expressed at low levels. The gating also allowed inclusion of other cells like non-$T_{FR}$ Treg cells, which would be useful for comparative analyses. The use of droplet-based single-cell RNA-Seq, which typically sequences thousands of cells in a single run assured that $T_{FR}$ cells would stochastically be represented in the data despite the permissive CXCR5 and PD-1 gating thresholds. We applied a standard 10x quality control and data processing pipeline as described above to quantify the transcriptome of 12,628 cells, which we subsequently clustered with the unsupervised SLM algorithm (291).

For each of the cells, we computed quantitative signatures of T cell identity (111) and visualized them with t-SNE plots. We identified 4 pertinent clusters of interest, one corresponding to Treg cells with enrichment in both Treg and $T_{FH}$ signatures (**Figure 2.1**;
Se_2_Treg/TFR in green), one corresponding to Treg cells with no TFH signature enrichment (Figure 2.1; Se_4_Treg in purple) and two corresponding to TFH cells (Figure 2.1; Se_3_TFH and Se_7_TFH in orange and pink). The signature derived from differential expression in population RNA-seq of sorted TFR and TFH cells (CD19^CD4^CXCR5^PD-1^FoxP3^+ and CD19^CD4^CXCR5^PD-1^FoxP3^- respectively) from draining lymph nodes of Foxp3^{RES-cGFP} reporter mice immunized with NP-OVA is also mostly enriched in the Se_2_Treg/TFR cluster. Next, we verified the identification of these clusters through genes differentially enriched in them. As expected, Se_2_Treg/TFR and Se_4_Treg clusters express Foxp3 while TFH-associated genes, including Cxcr5, Pdcd1, Icos, Maf and, to a less extent, Bcl6, are only enriched in the Se_2_Treg/TFR cluster. It is likely that the Se_2_Treg/TFR cluster not only includes TFR cells but also non-TFR effector Treg cells with enriched expression of Prdm1 (encoding BLIMP-1) and Tnfrsf18 (encoding GITR). The two TFH clusters, on the other hand, are enriched in the TFH marker genes with minimal Foxp3 enrichment (Figure 2.2).
Figure 2.1 t-SNE visualization of 10x single-cell transcriptomes. Four relevant clusters of interest (left) are shown whereas the grey dots correspond to cells belonging to other clusters. The other three panels present values of computational signatures allowing one to assign identities to the clusters.
**Figure 2.2** Genes that are differentially expressed between the clusters in **Figure 2.1** are shown. All of the genes, except for the ones in red, are the top differentially expressed genes in the corresponding clusters. The genes in red, on the other hand, are assigned manually as they are known T<sub>FH</sub>/Treg marker genes, as well as Fgl2.
To screen for potential novel T<sub>FR</sub> effector molecules, we sought genes that are a) preferentially detected in the Se_2_Treg/T<sub>FR</sub> cluster compared with the two T<sub>FH</sub> clusters b) preferentially detected in the Se_2_Treg/T<sub>FR</sub> cluster compared with the Se_4_Treg cluster and c) associated with an extracellular secreted product (GO:0005576). Through these criteria, we identified Fgl2 among the top genes (Figure 2.3). Additionally, we investigated the population RNA-seq data on T<sub>FR</sub> cells and T<sub>FH</sub> cells (Figure 2.4) and confirmed that Fgl2 was among the top genes encoding secreted proteins differentially expressed by T<sub>FR</sub> cells when compared to T<sub>FH</sub> cells. qPCR results confirmed that T<sub>FR</sub> cells expressed a high level of Fgl2 in comparison with T<sub>FH</sub> cells while non-T<sub>FR</sub> Treg cells also expressed high levels of Fgl2 as described previously (117). On the other hand, naïve T cells, total CD19<sup>+</sup> CD4<sup>+</sup> B cells and germinal center B cells expressed low levels of Fgl2 if any (Figure 2.5).
Figure 2.3 (a) Markers of T<sub>FR</sub> cells should be enriched in Treg/T<sub>FR</sub> cluster compared with both T<sub>TH</sub> and non-T<sub>FR</sub> Treg clusters. Each dot corresponds to a gene associated with an extracellular secreted product (GO:0005576). Its x- and y-values are BH-adjusted p-values for hypergeometrically testing whether detections of the gene are enriched in the given comparisons. (b) Fgl2 expression in the 4 clusters. Each dot corresponds to a cell that express Fgl2.
**Figure 2.4** Heatmap showing genes encoding secreted proteins differentially expressed by $T_{FR}$ cells when compared to $T_{FH}$ cells.

**Figure 2.5** Taqman qPCR showing high expression of Fgl2 by $T_{FR}$ cells compared to $T_{FH}$ cells, naïve T cells, GC B cells and total B cells.
**Fgl2 directly binds B cells and T<sub>FH</sub> cells**

To test the hypothesis that Fgl2 is an effector molecule of T<sub>FR</sub> cells, we first investigated whether Fgl2 directly binds to B cells and T<sub>FH</sub> cells, as Fgl2 was previously demonstrated to bind to DCs, peritoneal macrophages and total splenic B cells (236, 292). Using recombinant Fgl2 protein with a His tag, we showed that Fgl2 also binds in a dose-dependent fashion to total CD19<sup>+</sup> B cells in draining lymph nodes of NP-OVA/CFA immunized mice for 7 days (Figure 2.6). Further analysis of B cell subsets revealed preferential binding of Fgl2 to light-zone (LZ) germinal center B cells, defined as CD19<sup>+</sup>CD38<sup>-</sup>Fas<sup>+</sup>GL-7<sup>+</sup>CXCR4<sup>-</sup>CD86<sup>+</sup> cells (184) (Figure 2.7) The preferential binding to germinal center B cells, particularly LZ GC B cells, implies its relevance to T<sub>FR</sub> functions as suppressors of GCs. Fgl2 also preferentially binds to T<sub>FH</sub> cells, defined as CD19<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup> cells, in a dose-dependent fashion (Figure 2.8).

![Fgl2 Binding (Total B cells)](image)

**Figure 2.6** Draining lymph node cells or bone marrow cells from NP-OVA/CFA immunized mice for 14 days were stained and treated with recombinant Fgl2 with His-tag followed by secondary anti-His antibodies with fluoroflores (APC or PE). Fgl2 binds to total B cells.
(CD19+CD4+) from NP-OVA/CFA immunized mice for 7 days with the titration curve showing a dose-dependent binding.

**Figure 2.7** Fgl2 binds to different B cell subsets gated as followed: total GC B cells (CD19+CD38+Fas+GL-7+), DZ GC B cells (CD19+CD38+Fas+GL-7+CXCR4+CD86+), LZ GC B cells (CD19+CD38+Fas+GL-7+CXCR4+CD86+), memory B cells (CD19+CD38+CD138+), plasma cells (CD19+CD38+CD138+) and naïve B cells (CD19+IgM+IgD+).

**Figure 2.8** Fgl2 preferentially binds to T_{FH} cells (CD19+CD4+Foxp3+CXCR5+PD-1+) with the titration curve showing a dose-dependent binding.
Figure 2.9 Fgl2 binding was partially abolished in both LZ GC B cells and T<sub>FH</sub> cells in the presence of Fc block.

Figure 2.10 Expression of Fcgr2b and Fcgr3 mRNA were assessed by Taqman qPCR in sorted T<sub>FH</sub> cells, T<sub>FR</sub> cells and GC B cells from mice immunized with NP-OVA/CFA for 7 days.
Fgl2 has been reported to bind two receptors: FcγRIIb and FcγRIII (236). Since Fgl2 can bind to both B cells and T_{FH} cells, we studied if the binding was dependent on the two receptors and how the receptors are expressed. Treatment with Fc block, which antagonizes both receptors, partially suppressed Fgl2 binding both on LZ GC B cells and T_{FH} cells (Figure 2.9). Moreover, we found that, at the RNA level, Fcgr2b (encoding FcγRIIb) is highly expressed on GC B cells and, at a lower level, on T_{FH} cells while Fcgr3 (encoding FcγRIII) is expressed on T_{FR} cells and, to a lower extent, on GC B cells (Figure 2.10). We were unable to specifically differentiate between the two receptors at the protein level as available antibodies for flow cytometry were not able to discriminate between FcγRIIb and FcγRIII.

**Fgl2 directly regulates B cells**

Next, we analyzed the effects of Fgl2 on B cells and T_{FH} cells. We cultured sorted total splenic CD19^+CD4^- B cells from non-immunized mice in different conditions in the presence of Fgl2 and found that Fgl2 limits B cells survival and proliferation under anti-IgM condition but not in LPS and anti-CD40 conditions (Figure 2.11).
**Figure 2.11** Total B cells (CD19⁺CD4⁺) from wild-type mice were cultured under different conditions with or without Fgl2 treatment. Viable cells were analyzed by flow cytometry. Proliferation was measured using CFSE.

To investigate if Fgl2 influences B cell class-switch recombination (CSR), we cultured sorted total CD19⁺CD4⁺ splenic B cells from non-immunized mice in cytokine-polarizing conditions in the presence of LPS with or without recombinant Fgl2 for 4 days. The presence of recombinant Fgl2 inhibited IgG1 and IgE CSR under IL-4-polarizing conditions, but modestly
enhanced IgG2b under IFN-\(\gamma\)-polarizing conditions as assayed by intracellular staining and cytometric bead array. Similar results were also observed when total CD19\(^+\)CD4\(^-\) splenic B cells from mice immunized with NP-OVA/CFA for 7 days were used (Figure 2.12). These findings suggested that B cell CSR regulation by Fgl2 is context-dependent and isotype-selective. The impact of Fgl2 on B cell CSR was not dependent solely on either Fcgr2b or Fcgr3 as Fgl2 still retained its effects on single-knockout B cells. However, while we could not generate double-knockout mice due to the close proximity of loci encoding the two receptors, the presence of Fc block abrogated the effects of Fgl2 on B cell CSR but never reached the wild-type level, suggesting that there may be additional receptors through which Fgl2 must act (Fig. 2.12).
Figure 2.12 Fgl2 regulates B cell CSR *in vitro*. Effect of Fgl2 on B cell CSR: *In vitro* class switching assay. Sorted total B cells (CD19⁺CD4⁻) nonimmunized wild-type, FcγRIIb⁻/⁻ or FcγRIII⁻/⁻ mice for 7 days were cultured in LPS + IL-4 (IgG1 and IgE) or LPS + IFN-γ (IgG2b)
for 4 days in the presence or absence of Fc block. Switched isotypes were detected by flow cytometry.

**Fgl2 directly regulates T\textsubscript{FH} cells**

To test the effects of Fgl2 on T\textsubscript{FH} cells, we sorted T\textsubscript{FH} cells and cultured them with plate-bound anti-CD3 and anti-CD28. The addition of Fgl2 suppressed production of most secreted cytokines tested, including IFN-\(\gamma\), IL-2, IL-4, IL-10, IL17A, IL-21 and IL-13 *in vitro* (Figure 2.13a). However, these data should be interpreted with caution as expression of T\textsubscript{FH}-associated genes, including Bcl6, Maf, Cxcr5 and Icos, was lost upon *in vitro* activation by anti-CD3/anti-CD28 in the absence of B cells (Figure 2.13b). The addition of total CD19\(^+\)CD4\(^-\) B cells from the same immunized mice we harvested T\textsubscript{FH} cells in the T\textsubscript{FH}/B cell co-culture in the presence of soluble anti-CD3 and anti-IgM (279, 281) resulted in suppression of secreted IL-13 and IL-5, but not IFN-\(\gamma\), in the presence of soluble Fgl2 (Figure 2.14). B cell survival in the presence of T\textsubscript{FH} cells was not affected (Figure 2.15a) unless B cells and T\textsubscript{FH} cells were from immunization with different antigens (Figure 2.15b). This selective suppression of type-2 cytokines was associated with a selective decrease in IgG1 production (Figure 2.14). However, whether the decrease in IgG1 production was due to the altered cytokine profile of the T\textsubscript{FH} cells is unclear, as Fgl2 was demonstrated earlier to directly suppress IgG1 CSR on B cells (Figure 2.12).
Figure 2.13 (a) Sorted T<sub>FH</sub> cells were cultured under plate-bound anti-CD3/anti-CD28 with Fgl2 or PBS control. Culture media samples were collected at different time points and cytokines were analyzed by LegendPlex. (b) Taqman gene expression analysis on ex vivo T<sub>FH</sub> cells vs. T<sub>FH</sub> cells after the culture as in (a) for 96 hours.
**Figure 2.14** Effects of Fgl2 on T\(_{FH}\)/B cell co-culture: sorted T\(_{FH}\) cells were co-cultured with sorted total B cells from the same immunized mice in the presence of soluble anti-CD3 and anti-IgM with or without Fgl2 for 3 days. Cytokines were detected by bead-based LEGENDPlex kit and antibody isotypes were detected by bead-based mouse immunoglobulin isotyping kit.

**Figure 2.15** (a) Viability of B cells from T\(_{FH}/T_{FR}/B\) co-culture assay measured by flow cytometry. (b) Viability of B cells from T\(_{FH}/B\) co-culture assay from mice immunized with either NP-OVA/CFA or KLH/CFA for 7 days with or without Fgl2 treatment.
Fgl2 regulates antibody responses through \( T_{FH} \) cells \textit{in vitro}

To investigate whether the role of Fgl2 in regulating antibody responses is dependent on \( T_{FH} \) cells, we used a well characterized co-culture system previously described (281). As \( T_{FH} \) cells only express Fc\( \gamma \)RIIb but not Fc\( \gamma \)RIII, we tested whether the Fgl2 would regulate IgG1 suppression through \( T_{FH} \) cells deficient in Fc\( \gamma \)RIIb. Addition of Fgl2 to wild type B cells and \( T_{FH} \) cultures resulted in significant inhibition of IgG1 production. Although co-culturing wild-type B cells with Fc\( \gamma \)RIIb\(^{-/-}\) \( T_{FH} \) cells showed no significant difference in IgG1 level in the absence of Fgl2, the suppression of IgG1 by exogenous Fgl2 was partially rescued when Fc\( \gamma \)RIIb\(^{-/-}\) \( T_{FH} \) cells were present in the culture (Figure 2.16), suggesting that the Fc\( \gamma \)RIIb receptor expression on \( T_{FH} \) cells can regulate IgG1 CSR in the presence of exogenous Fgl2.

**Figure 2.16** Wild-type \( T_{FH} \) cells or Fc\( \gamma \)RIIb\(^{-/-}\) \( T_{FH} \) cells were co-cultured with wild-type B cells from immunized mice in cells in the presence of soluble anti-CD3 and anti-IgM with or without Fgl2 for 3 days. IgG1 in B cells was detected by flow cytometry. Secreted IgG1 in the media was detected by bead-based mouse immunoglobulin isotyping kit.
To further investigate potential downstream molecules of T\textsubscript{FH} cells that modulate IgG1 CSR in response to Fgl2, we re-sorted T\textsubscript{FH} cells after the co-culture with B cells and Fgl2 and analyzed gene expression profiling using the Nanostring platform. We not only failed to see inhibition of T\textsubscript{FH}-related genes, including Bcl6, Pdcd1, and IL-21, but these genes were in fact up-regulated as compared with T\textsubscript{FH} cells co-cultured in the absence of Fgl2. However, there was induction of genes associated with T\textsubscript{FH} functional suppression, including Ctl4 and Prdm1. The induction of Prdm1 was also confirmed by qPCR (Figure 2.17). The effect of Fgl2 on Prdm1 expression in T\textsubscript{FH} cells was also observed in vivo as Prdm1 was among the top genes differentially expressed genes when expression analysis was undertaken between wild-type and Fgl2\textsuperscript{-/-} derived T\textsubscript{FH} cells and the reduction in Prdm1 expression in T\textsubscript{FH} cells from Fgl2\textsuperscript{-/-} mice was confirmed by qPCR (Figure 2.18). Prdm1 induction by Fgl2 in T\textsubscript{FH} cells was dependent on Fc\gammaRIIb on the cells as Fc\gammaRIIb-deficient T\textsubscript{FH} cells failed to upregulate Prdm1 in response to Fgl2 treatment as measured by qPCR from T cells that had been re-sorted from T\textsubscript{FH}/B cell co-culture experiments (Figure 2.19).
Figure 2.17 (a) Sorted T<sub>FH</sub> cells were co-cultured with sorted total B cells from immunized mice in the presence of soluble anti-CD3 and anti-IgM with or without Fgl2 for 3 days. T cells were then re-sorted and subjected to NanoString gene expression profiling. The genes shown were filtered based on differential expression over 2 folds while p < 0.05. (b) Prdm1 expression was evaluated by Taqman qPCR.
Figure 2.18 (a) T_{FH} cells were sorted from wild-type or Fgl2^{-/-} mice and were subjected to RNA-seq. Some of the top differentially-expressed genes were shown in the volcano plot. Prdm1 was among the top genes differentially expressed and its expression was confirmed by Taqman qPCR in (b).

Figure 2.19 Wild-type T_{FH} cells or FcγRIIb^{-/-} T_{FH} cells were co-cultured with wild-type B cells from immunized mice in cells in the presence of soluble anti-CD3 and anti-IgM with or without Fgl2 for 3 days. Prdm1 expression was evaluated by Taqman qPCR.
To test if Prdm1 is downstream of Fgl2 in T<sub>FH</sub> cells, we co-cultured wild-type B cells with Prdm1-deficient T<sub>FH</sub> cells. Notably, T<sub>FH</sub> cells deficient in both Prdm1 copies (Prdm1<sup>−/−</sup>) failed to induce IgG1 in B cells (Figure 2.20) even though Prdm1 has been shown to antagonize Bcl6 (39). Thus, we hypothesized that such T<sub>FH</sub> cell function must require some level of Prdm1 expression, suggesting that the regulation by Prdm1 is dependent on the level of Prdm1 expression. In fact, mice deficient only in one copy of Prdm1 were previously shown to possess a phenotype distinct from those deficient in both copies (293). Using T<sub>FH</sub> cells from mice deficient in one copy of Prdm1 (Prdm1<sup>−/+</sup>), we found that these T<sub>FH</sub> cells were as capable of inducing IgG1 in B cells as the wild-type control T<sub>FH</sub> cells, and that the reduction in Prdm1 gene number conferred significant resistance to Fgl2-mediated suppression of IgG1 B cells upon Fgl2 treatment (Figure 2.21).

![Figure 2.20](image-url) Control T<sub>FH</sub> cells (Prdm1<sup>fl/fl</sup> or Prdm1<sup>−/−</sup> with CD4cre<sup>+</sup>) or Prdm1<sup>−/−</sup> (Prdm1<sup>fl/fl</sup> with CD4cre<sup>+</sup>) T<sub>FH</sub> cells were co-cultured with wild-type B cells from immunized mice in cells in the presence of soluble anti-CD3 and anti-IgM with or without Fgl2 for 3 days. IgG1 in B cells was detected by flow cytometry. Secreted IgG1 in the media was detected by bead-based mouse immunoglobulin isotyping kit.
Figure 2.21 Control TFH cells (Prdm1^{+/+} or Prdm1^{+/−} with CD4cre<Elo> or Prdm1^{+/−} (Prdm1^{−/−} with CD4cre<Elo>) TFH cells were co-cultured with wild-type B cells from immunized mice in cells in the presence of soluble anti-CD3 and anti-IgM with or without Fgl2 for 3 days. IgG1 in B cells was detected by flow cytometry. Secreted IgG1 in the media was detected by bead-based mouse immunoglobulin isotyping kit.

As Prdm1 was previously shown to be involved in regulating expression of co-inhibitory gene module (133), we also asked if Fgl2 had effects on checkpoint molecules on TFH cells. We found that Fgl2 induced upregulation of multiple co-inhibitory, check-point molecules, including PD-1, TIM-3, LAG-3 and TIGIT on TFH cells co-cultured with B cells, by measuring both mRNA and protein levels (Figure 2.22). The results suggest that Fgl2 can partially suppress IgG1 in B cells through modulation of Prdm1 level in TFH cells, potentially antagonizing Bcl6 (39), in an FcγRIIb-dependent fashion while additionally further inducing t expression co-inhibitory molecules on TFH cells.
**Figure 2.22** Sorted $T_{FH}$ cells were co-cultured with sorted total B cells from immunized mice in the presence of soluble anti-CD3 and anti-IgM with or without Fgl2 for 3 days. (a) Resorted T cells were subjected to Taqman qPCR to measure Prdm1, Havcr2, Lag3 and Tigit expression. (b) and (c) Protein expression of PD-1, TIM-3, LAG-3 and TIGIT were detected by flow cytometry.
Figure 2.22 (continued)

(a) Graphs showing relative expression of different markers (PDcd1, Havor2, Lag3, Tigit) in WT Th and WT Th + Fg2 conditions.

(b) Flow cytometry plots gated on CD19-CD4+ cells, showing expression levels of PD-1, TIM-3, LAG-3, and TIGIT in different conditions.

(c) Bar charts indicating the percentage of CD4+CD19+ cells expressing PD-1 and TIM-3 in various conditions.
**Fgl2 regulates antibody responses *in vivo***

In order to determine if Fgl2 modulates antibody responses *in vivo*, we analyzed antibody production in Fgl2-deficient mice. 20-week-old Fgl2−/− mice had elevated total serum IgG1, IgG2a, IgA and IgE spontaneously (**Figure 2.23**). We also analyzed Peyer’s Patches (PP) where T\textsubscript{FH} cells and GCs are present at steady state and we found that Fgl2−/− mice had increased PP IgA\textsuperscript{+} B cells, GC B cells, T\textsubscript{FH} cells and T\textsubscript{FR} cells, but a decreased total number of Treg cells (which include T\textsubscript{FR} cells) (**Figure 2.24**). These findings suggested that antibody responses at steady state were dysregulated with age in the absence of Fgl2 *in vivo*.

**Figure 2.23** Serum antibody isotypes in 20-week-old wild-type and Fgl2−/− mice were detected by bead-based mouse immunoglobulin isotyping kit and ELISA (for IgE). The mouse
immunoglobulin isotyping kit provides no standard curve assessment so the results should be considered more as qualitative.

![Image of immunological analysis](image)

**Figure 2.24** Peyer’s Patches of 20-month-old wild-type and FgI2⁻/⁻ mice were immunophenotyped by flow cytometry for IgA⁺ B cells, GC B cells, T<sub>FH</sub> cells, T<sub>FR</sub> cells and total Treg cells. Free fecal IgA was measured by ELISA.
Next we wanted to see if Fgl2 could affect antigen-specific responses. We immunized wild-type and Fgl2\(^{-/-}\) mice with NP-OVA emulsified in CFA. At day 21, we detected significantly enhanced NP-specific IgG1 but not NP-specific IgG2b by ELISA in Fgl2\(^{-/-}\) mice. Interestingly, examination at an earlier time point (day 10) when GC B cells and T\(_{FH}\) cells were still present showed no significant differences in the frequencies of GC B cells (Figure 2.25).

**Figure 2.25** Wild-type and Fgl2\(^{-/-}\) mice were immunized with NP-OVA (s.c.) in CFA for 21 days and NP-specific isotypes were detected by ELISA. GC B cells were analyzed after 14 days with flow cytometry after the same immunization condition.

**Fgl2 from T\(_{FR}\) cells regulates antibody responses in vitro and in vivo**

Because Fgl2 affected the numbers of both T\(_{FH}\) cells and T\(_{FR}\) cells *in vivo* and Fgl2 may come from other cellular sources including conventional Treg cells, we utilized co-culture and transfer experiments to study the contribution of Fgl2 from T\(_{FR}\) cells. To address whether Fgl2 from T\(_{FR}\) cells modulates antibody responses *in vitro*, sorted total CD19\(^+\) B cells from the immunized mice were co-cultured with T\(_{FH}\) cells and wild-type or Fgl2\(^{-/-}\) T\(_{FR}\) cells in the presence of soluble anti-CD3 and anti-IgM antibodies for 3 days. We used Fgl2\(^{-/-}\) B cells in this
system to make sure that the main source of Fgl2 will come from T_{FR} cells as B cells could produce some level of Fgl2 (Figure 2.25). As expected, the addition of wild-type T_{FR} cells suppressed IgG1 CSR as shown by intracellular IgG1 staining and Ig bead array on secreted IgG1 while using Fgl2^{-/-} T_{FR} cells partially rescued it. The addition of exogenous Fgl2 also further suppressed IgG1 CSR in all conditions (Figure 2.26). Non-T_{FR} Treg cells, which express Fgl2 (Figure 2.5), did not significantly suppress IgG1 CSR and Fgl2 depletion in non-T_{FR} Treg cells resulted in no significant difference in the suppression (Figure 2.27), suggesting the presence of T_{FR}-specific effects that render Fgl2 functional at physiological concentration. Such findings demonstrated that Fgl2 from T_{FR} cells suppress IgG1 responses in vitro.

![Graphs showing IgG+ B cells and secreted IgG1](image)

**Figure 2.26** In vitro co-culture assay. 50,000 Fgl2^{-/-} sorted total CD19^{+} B cells from immunized mice were co-cultured with 30,000 T_{FH} cells and 3,000 wild-type or Fgl2^{-/-} T_{FR} cells in the
presence of soluble anti-CD3 and anti-IgM antibodies. Recombinant Fgl2 was added in the conditions specified.

**Figure 2.27** *In vitro* co-culture assay with the same settings as in **Figure 2.26** while conditions with 3,000 wild-type or Fgl2<sup>−/−</sup> non-T<sub>FR</sub> Treg cells were included.

Next we sought to investigate if Fgl2 from T<sub>FR</sub> cells regulates antibody responses *in vivo*. Sorted T<sub>FH</sub> cells and T<sub>FR</sub> cells were acquired from mice immunized 7 days earlier with NP-OVA/CFA. Wild-type T<sub>FH</sub> cells were co-transferred with either wild-type T<sub>FR</sub> cells or Fgl2<sup>−/−</sup> T<sub>FR</sub>
cells into CD28−/− recipient mice, which lacked endogenous T_{FH}/T_{FR} cells as previously described (147, 279). The recipient mice, along with no-transfer controls, were then immunized with NP-OVA/CFA. At day 21, serum NP-specific IgG1 levels were significantly enhanced in mice receiving Fgl2−/− T_{FR} cells. GC B cells, T_{FH} cells and T_{FR} cells were analyzed on day 7 after recall immunization. The frequency of NP-specific GC B cells in mice receiving Fgl2−/− T_{FR} cells was slightly but significantly elevated while the frequency of T_{FH} cells and T_{FR} cells was not significantly different (Figure 2.28). Collectively, the data suggested that Fgl2 from T_{FR} cells regulates IgG1 and GC responses \textit{in vivo} without affecting gross T_{FH}/T_{FR} cell frequencies.
**Figure 2.28** Sorted 50,000 WT or Fgl2−/− CD19−CD4+CXCR5+PD1+ T cells (T_{FH} + T_{FR} cells) were transferred into CD28−/− mice. The mice were then immunized with NP-OVA/CFA for 14 days and NP-specific IgG1 was detected by ELISA. After 30 days, recall responses were induced and the cellular composition of inguinal lymph nodes was analyzed by flow cytometry.

**Fgl2 is required for autoantibody controls**

We then asked whether NP-OVA/CFA immunization alone would be sufficient to induce autoreactive B cells in young Fgl2−/− mice as influenza infection was previously shown to induce anti-dsDNA antibodies in mice specifically deficient in T_{FR} cells (149). ELISPOT results showed that Fgl2−/− mice and CD28−/− control mice, which completely lacked both T_{FH} and T_{FR} cells, had a significant increase in inguinal lymph node anti-dsDNA (total Ig) antibody-secreting cells (ASCs) with the peak at day 21 before the level started to go down while only wild-type and Fgl2−/− mice, but not CD28−/− control mice, have elevated inguinal lymph node anti-NP-OVA (total IgG) ASCs starting from day 7. Moreover, no significant differences in systemic levels of autoantibodies were observed as shown by the level of serum anti-dsDNA and anti-nuclear antibodies (ANA) (**Figure 2.29**). The data suggested that while Fgl2−/− mice still have functional total IgG ASCs against a foreign antigen despite some alterations in IgG isotypes demonstrated earlier, they failed to control local and transient expansion of autoreactive B cells during inflammation. This enhancement of autoreactive B cell responses strongly supports Fgl2 as a T_{FR} effector molecule that controls autoimmunity.
Figure 2.29 Wild-type and Fgl2⁻/⁻ mice were immunized by NP-OVA/CFA (with additional heat killed dried *Mycobacterium tuberculosis*). Inguinal lymph nodes were harvested at the time points indicated and the cells were subjected to ELISPOT assay to detect dsDNA Ig and NP-OVA IgG ASCs. Serum samples from the mice at the time points indicated were subjected to ANA and anti-dsDNA ELISA.

To test if Fgl2 from TᵢFR cells was relevant to the phenotype we saw in Fgl2⁻/⁻ mice, we asked if transfer of TᵢFR cells into CD28⁻/⁻ mice, which lacked TᵢFR cells and failed to control expansion of anti-dsDNA ASCs, would reverse the phenotype and whether the reversion was
Fgl2-dependent. In fact, transfer of 10,000 wild-type T\(_{FR}\) cells into CD28\(^{-}\) mice significantly suppressed expansion of anti-dsDNA ASCs upon NP-OVA/CFA immunization as measured by ELISPOT on day 21. However, the transfer of Fgl2\(^{-}\) T\(_{FR}\) cells in the same scheme resulted in significantly inferior suppression of such expansion (Figure 2.30), suggesting that Fgl2 from T\(_{FR}\) cells can at least partially suppress autoreactive B cells in vivo.

![dsDNA Ig ELISPOT](image_url)

**Figure 2.30** Sorted 10,000 WT or Fgl2\(^{-}\) CD19\(^{-}\)CD4\(^{-}\)CXCR5\(^{+}\)PD1\(^{+}\)Foxp3\(^{+}\) T\(_{FR}\) cells were transferred into CD28\(^{-}\) mice. The mice were then immunized with NP-OVA/CFA (with additional heat killed dried *Mycobacterium tuberculosis*) for 21 days and dsDNA Ig ASCs were detected by ELISPOT

Aged Fgl2\(^{-}\) mice (7-12 months) deficient in Fgl2 have previously been shown to have impaired Treg function and develop glomerulonephritis (227). However, autoantibody levels have not been investigated. To assess whether there were active autoreactive B cells in aged 12-
month-old Fgl2<sup>−/−</sup> mice with no external perturbation, we performed ELISPOT experiments to
detect anti-dsDNA ASCs using total splenocytes from aged Fgl2<sup>−/−</sup> mice and age-matched wild-
type controls and found that aged Fgl2<sup>−/−</sup> mice had elevated splenic anti-dsDNA (total Ig) ASCs.
To determine if the elevation of autoantibodies was systemic, we measured serum antibodies and
found elevated levels of anti-dsDNA IgG1 and ANA in aged Fgl2<sup>−/−</sup> mice as compared to age-
matched wild-type controls (Figure 2.31). A more comprehensive detection was performed
using Lupus-associated autoantigen microarrays. We observed elevation of multiple
autoantibodies against Lupus-associated autoantigens, including ssDNA, alpha elastin, β2
glycoprotein, dsDNA, U1 snRNP and collagen x, in aged Fgl2<sup>−/−</sup> mice, and the distribution of
IgM/IgG isotypes also reflected disease severity based on histological data (Figure 2.32).

![Figure 2.31](image1.png)

**Figure 2.31** Splenocytes from 12-month-old wild-type and Fgl2<sup>−/−</sup> mice were subjected to
dsDNA Ig ELISPOT. Anti-dsDNA IgG1 and ANA in sera from 12-month-old wild-type and
Fgl2<sup>−/−</sup> mice were measured by ELISA.
Figure 2.32 An array of lupus IgM and IgG autoantibodies were measured in sera from 12-month-old wild-type (n = 6) and Fgl2<sup>-/-</sup> mice (n = 9) using autoantigen microarray. Fgl2<sup>-/-</sup> mice with mild diseases (n = 5) had either normal histology or mild dermatitis while mice with high, severe diseases (n = 4) had inflammation in multiple organs, including severe dermatitis, reactive changes in the spleen, ileitis, enlarged Peyer's Patches and increased lymphoid clusters in lung.

Aged Fgl2<sup>-/-</sup> Mice Developed Inflammatory, Lupus-like Autoimmunity
Forty-two percent of the aged, 12-month-old, Fgl2−/− mice in our cohort spontaneously developed a skin-associated phenotype that included patches of hair loss, hyperkeratosis and dermatitis (Figure 2.33). Histological analysis of the skin showed signs of surface ulceration, hyperkeratosis, elongation of rete ridges, dermal scarring, epidermolysis, follicular plugging (Figure 2.34, arrow) and basal cell discohesion (Figure 2.34, arrow). Infiltration of T and B cells into the skin was also observed (Figure 2.34). 18% of the aged mice also had spontaneous germinal center cell phenotype in spleens (Figure 2.33 and 2.35). The results suggested that the loss of Fgl2 led not only to systemic elevation of Lupus-associated autoantigens but also clinical manifestations of inflammatory diseases.

<table>
<thead>
<tr>
<th>Skin-associated phenotypes (external; hairless patches to dermatitis)</th>
<th>Aged WT (%/rats)</th>
<th>Aged Fgl2−/− (%/rats)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperkeratosis, dermal scarring</td>
<td>0/9 (0%)</td>
<td>5/19 (26%)</td>
</tr>
<tr>
<td>Skin infiltration of T/B cells</td>
<td>0/9 (0%)</td>
<td>3/19 (16%)</td>
</tr>
<tr>
<td>Reactive germinal centers</td>
<td>0/9 (0%)</td>
<td>3/19 (16%)</td>
</tr>
<tr>
<td>Skin infiltration of mast cells, activated macrophages</td>
<td>0/9 (0%)</td>
<td>1/19 (5%)</td>
</tr>
</tbody>
</table>

Figure 2.33 A portion of aged, 12-month-old, Fgl2−/− mice spontaneously developed skin disease with varied severity.
Figure 2.34 Histological analysis shows signs of surface ulceration, hyperkeratosis, elongation of rete ridges, dermal scarring, epidermolysis, follicular plugging (arrow) and basal cell discohesion (arrow). Infiltration of T cells and B cells was also observed.
Figure 2.35 Cellular composition from mice with spontaneous reactive splenic GCs showed increased in GC B cells and T\textsubscript{FH} cells.

Discussion

We have used single-cell transcriptome analysis and population RNA-seq to identify Fgl2 as a top-ranking gene in follicular regulatory T cells. Moreover, we showed that Fgl2 is preferentially expressed in T\textsubscript{FR} cells as compared to T\textsubscript{FH} cells, naïve T cells and GC B cells and that it is critical in controlling type-2 antibody responses and autoreactive B cell responses that
spontaneously arise during inflammation as well as when the mice age. Aged Fgl2\(^{-/-}\) mice develop spontaneous skin inflammation and Lupus-like phenotypes with elevation of autoantibodies, highlighting the critical role of Fgl2 in regulating autoimmunity. We showed that Fgl2 directly acts on B cells and T\(_{FH}\) cells and regulates antibody production and class switch recombination, and T\(_{FR}\) derived Fgl2 is critical for these processes, thus demonstrating that Fgl2 is a T\(_{FR}\) effector molecule both \textit{in vitro} and \textit{in vivo}.

T\(_{FR}\) cells have been shown to express Treg-associated genes along with T\(_{FH}\)-associated ones (146-148). Our data, based on analysis of the transcriptome at the resolution of single cells through a clustering scheme, showed that T\(_{FR}\) cells are similar to effector Treg cells as they are in the same cluster in tSNE. This finding is consistent with earlier observations on Treg cells (156). Our analysis, however, also took well-defined T\(_{FH}\) cells into account. These results, therefore, demonstrate that T\(_{FR}\) cells are still fundamentally Treg cells that express some T\(_{FH}\)-associated genes rather than simply Treg/T\(_{FH}\) hybrid cells.

The suppression of type-2 antibody responses by Fgl2 was unexpected as Fgl2 was previously shown to suppress effector T\(_{H}\)1 and T\(_{H}\)17 but not T\(_{H}\)2 cells (117). However, our data also demonstrated that the effects of Fgl2 are, in fact, context and cell type-dependent (Figure 2.13a and 2.14). The presence of B cells, which is required for maintaining the T\(_{FH}\) phenotype (Figure 2.13b), dictates this bias, as in the absence of B cells we observed extensive cytokine suppression effects in non-T\(_{FH}\) effector cells cultured \textit{in vitro}. It is important to note that even though Fgl2 suppresses type-2 antibody responses directly on B cells, its effect can be exerted through T\(_{FH}\) cells via the Fc\(_{Y}\)RIIb (Figure 2.16), suggesting a nuanced mechanism. Thus, it is not surprising that the contexts where Fgl2 is expressed and the cells it acts on could lead to different effector outcomes.
We have shown that Fgl2 induces Prdm1 in T_{FH} cells by an FcγRIIb-dependent mechanism and that the pathway exerts inhibition of T_{FH}-mediated IgG1 CSR on B cells. Expression of Prdm1 is potentially critical at multiple levels including antagonization of Bcl6 (39) and induction of a panel of check-point molecules (PD-1, TIM-3, LAG-3 and TIGIT). It is possible that dysregulation of check-point molecule expression might account for aspects of the autoimmune phenotypes we observed in aged Fgl2-deficient mice. In fact, it was recently observed that germline HAVCR2 (encoding TIM-3) mutations were associated with subcutaneous panniculitis-like T cell lymphomas with some patients exhibiting increased IgE levels and positive anti-DNA antibodies (294).

While Fgl2 produced by Treg cells has been previously shown to inhibit effector T cell responses, its functions based on T_{FR} cells have not been studied. Here we demonstrated the relevance of Fgl2 in T_{FR} cells in regulating antibody responses both in vitro and in vivo. In addition, mice deficient in Fgl2, unlike total Treg-deficient mice with severe multi-organ inflammation at young age (295, 296), were generally healthy at a young age while immunization led to altered isotypes against the immunizing antigen. Notably, such immunization also resulted in local induction of autoreactive B cells producing anti-dsDNA. The phenotype was similar to that observed in T_{FR}-deficient mice (149, 150), which exhibit subtle alterations in humoral responses against foreign antigens and only develop autoimmunity at an older age or upon induction. These similarities, thus, strongly support the relevance of T_{FR}-derived Fgl2 in the aspects observed.

Recent studies have implicated T_{FR} cells in limiting autoimmunity (149, 150). Our observations that NP-OVA immunization was sufficient to induce transient and local induction of autoreactive B cells in Fgl2-deficient mice and that the mice spontaneously developed
dermatitis and multiple types of autoantibodies against Lupus-associated autoantigens with age suggested that Fgl2 is an effector molecule used by T<sub>FR</sub> cells to control autoreactive B cells. T<sub>FR</sub> cells might directly interact with autoreactive B cells themselves as their TCR repertoire is skewed toward self-antigen (154). Our data, in addition, show that Fgl2 is only able to limit B cell survival and proliferation in the absence of cognate T<sub>FH</sub> help (Figure 2.15b) or another co-stimulatory pathway like TLR4 by LPS stimulation (Figure 2.11). This observation implies that, through Fgl2, T<sub>FR</sub> cells might specifically target autoreactive B cells due to their lack of help from cognate T<sub>FH</sub> cells. Moreover, dysregulation in co-stimulatory pathways such as TLR pathways that leads to autoreactive B cell dysregulation (297, 298) might be partially due to their resistance to T<sub>FR</sub> suppression.

The presence of autoantibodies against lupus-associated autoantigens in aged Fgl2<sup>−/−</sup> mice leads to the hypothesis that Fgl2 may be a relevant molecule in lupus and/or other humoral autoimmune diseases like Sjogren's syndrome. In fact, mice with FcγRIIb deficiency develop a spontaneous lupus-like disease with high titer autoantibodies and B cells from the mice can lead to the development of lupus-like disease (243). Furthermore, polymorphisms in both low-affinity Fc receptors known to bind to Fgl2 have been shown to be associated with susceptibility to SLE in humans (251, 253, 254, 256, 299). These studies not only underscore the role of these Fc receptors but may also suggest that the effects are partly due to loss of Fgl2 signaling.

Fgl2<sup>−/−</sup> mice have been reported previously to develop glomerulonephritis, which we did not observe in our cohort (227). However, we were able to see spontaneous germinal center cell phenotype in spleens in a small portion of the observed group. This suggests that additional environmental factors, including possible variation in the microbiome in our housing facility, likely modulate disease. As we hypothesize that the phenotype observed with age was dependent
on accumulation of immunological insults over a lifespan, the variability in the frequency and
the underlying natures of such insults, such as host-microbiome interactions, might result in
manifestation of different clinical phenotypes in Fgl2-deficient mice.

Taken together, our work uncovered Fgl2 as a $T_{FR}$ cell effector molecule that directly acts
on B cells and $T_{FH}$ cells. This finding provides a novel path for targeting antibody responses by
modulating $T_{FR}$ effector function, which could potentially be useful for vaccine development
and, through supplementation, for treating systemic autoimmune diseases.
Chapter 3

Linear Differentiation Trajectory of Follicular Helper T cells from Th1 Cells
Abstract

Follicular helper T (T_{FH}) cells are a subset of CD4 helper T cells that express the transcription factor Bcl-6 and mediate different aspects of germinal center responses. However, T_{FH} cells can also express transcription factors and cytokines associated with other CD4 helper T cells and the ontogeny of these Th-like T_{FH} cells has been a topic of debate. Through single-cell RNA-seq analysis of the cellular heterogeneity of draining lymph node T_{FH} cells derived from mice immunized with NP-OVA/CFA, we identified T_{FH} cells with weaker expression of T_{FH} signature genes and stronger expression of Th1 signature genes. In combination with the use of a T-bet fate-mapping mouse strain, we found that a significant portion of T_{FH} cells previously expressed T-bet and that these ex-T-bet\(^+\) T_{FH} cells still expressed residual Th1 cell-associated genes, supporting a linear differentiation trajectory in which these T_{FH} cells are derived from Th1 cells. Gene expression analysis also suggested STAT3 as a mediator of this Th1/T_{FH} cell linear differentiation trajectory and deletion of STAT3 in T-bet\(^+\) cells resulted in severe defects in the T-bet fate\(^+\) T_{FH} cell abundance. Taken together, the results support a linear differentiation trajectory in which Th1-like T_{FH} cells are derived from Th1 cells that later adopt T_{FH} gene effector modules.
Introduction

Follicular helper T (T<sub>FH</sub>) cells are specialized CD4 helper T cells that confer help to B cells in histologically distinct germinal centers (GCs) (36-38) and the identification of Bcl-6 as a T<sub>FH</sub> cell master regulator established them as a distinct T cell subset (39-41). While it was shown that T<sub>FH</sub> cells have significantly reduced expression of Th1-, Th2-, or Th17-related transcription factors and cytokines (36, 136), it has been demonstrated that CXCR5<sup>+</sup> T cells still produce IFN-γ (39, 137, 138), IL-4 (138-140) and IL-17 (86). Moreover, it was also shown that, Bcl6<sup>+</sup> lymph node cells isolated from mice immunized with KLH/CFA could simultaneously express IFN-γ or IL-4. Also, both Th1 and Th2 cells, but not Th17 cells, could be differentiated in vitro to become T<sub>FH</sub> cells. However, the expression of Th1/Th2 signature genes in Bcl6<sup>+</sup> cells was significantly reduced over time as Bcl6 expression increased (141). Interestingly, while some studies showed that transfer of in vitro differentiated Th1, Th2 or Th17 cells were able to support antibody responses (137, 139, 142), others showed that T<sub>FH</sub> differentiation in vivo did not depend on Th1, Th2 or Th17 differentiation pathways (136). Phenotypic overlaps between T<sub>FH</sub> cells and other Th cells might be important for T<sub>FH</sub>-mediated class-switch recombination to generate antibody isotypes by B cells, as IFN-γ was shown to induce class switching to isotypes like IgG2a in the context of viral infection (143-145) while IL-4 favors mouse IgG1/IgE in the context of parasitic infection (138) and IL-17 favors IgG2a and IgG3 (142). In addition, dysregulation of cytokine production by T<sub>FH</sub> cells was shown to be associated with autoimmunity as seen in a spontaneous Lupus model sanroque where T<sub>FH</sub> cells overproduce IFN-γ (300).

The phenotypic overlaps in T<sub>FH</sub> cells raises some questions about the relationship between T<sub>FH</sub> cells and other CD4 T cell subsets, as their signature transcriptional profiles do not appear to be entirely mutually exclusive. In fact, a key issue remaining to be addressed is
whether $T_{FH}$ cells that produce cytokines typical for other CD4 Th subsets \textit{in vivo} arise from a distinct $T_{FH}$ subset that acquires other effector modules (lineage model) or from other CD4 subsets that acquire secondary $T_{FH}$ effector modules (linear model). Here, using single-cell RNA-seq experiments in combination with T-bet fate reporter mice, we identified Th1-like $T_{FH}$ cells following NP-OVA/CFA immunization that correspond to $T_{FH}$ cells that previously express T-bet. Interestingly, while T-bet fate\textsuperscript{+} $T_{FH}$ cells are mostly T-bet\textsuperscript{+}, they still retain significant expression of Th1-associated genes at single cell level. The results demonstrate that these Th1-like $T_{FH}$ cells are derived from Th1 cells. Moreover, we identified STAT3 as a Th1/$T_{FH}$ differentiation switch and its genetic deletion in T-bet\textsuperscript{+} cells led to specific defects in T-bet fate\textsuperscript{+}, Th1-like $T_{FH}$ cells. Together, these findings revealed the presence of a linear differentiation trajectory for $T_{FH}$ cells as they can be derived from another CD4 T cell subset.

**Materials and Methods**

**Mice.** Wild-type C57BL/6J, Rosa\textsuperscript{LSLT\textasciitildeTomato}, STAT3\textsuperscript{β/β} and T-bet\textsuperscript{cre} mice were purchased from Jackson Laboratories. T-bet\textsuperscript{cre} mice were backcrossed to wild-type C57BL/6J mice for 10 generations in the lab. All mice were between 6 and 8 weeks of age at the time of experiments unless specific ages were mentioned in particular experiments. All mice used in each experiment were age-matched and gender-matched. The experiments were conducted in accordance with animal protocols approved by the Harvard Medical Area Standing Committee on Animals or BWH IACUC.
**Immunizations.** Mice were subcutaneously immunized with 100 μg NP-OVA (Biosearch Technologies) emulsified in Complete Freund's Adjuvant (BD) in the mouse flanks as previously described (279).

**Antibodies.** The following antibodies were used for surface staining: anti-CD4 (RM4-5), anti-CD19 (6D5), anti-CXCR5 biotin (2G8) and anti-PD-1 (RMP1-30). Secondary staining for biotinylated primary antibody was done using streptavidin (BioLegend). For intracellular staining samples were fixed with Fixation/Perm solution kit (BD) for intracellular Ig or FoxP3 Fix/Perm buffer set (eBioscience) for staining transcription factors according to manufacturer’s instruction. Samples were then intracellularly stained with FoxP3 (FJK-16S), anti-Bcl6 (K112-91) and anti-T-bet (4B10).

**Sorting.** Single cell suspensions were diluted in PBS supplemented with 3% FBS with 2mM EDTA. CD4⁺ cells were enriched by magnetic positive selection (Miltenyi Biotec). CD4⁺ enriched cells were then stained and doubly-sorted as specified in specific experiments. For plate-based single-cell RNA-seq, the cells were single cell-sorted into 96-well plates with 5 μL RLT buffer with 1% 2-mecaptoethanol and immediately frozen.

**Droplet-based single-cell RNA-Seq.** Inguinal lymph nodes from wild-type mice immunized with NP-OVA/CFA (s.c.) for 7 days were isolated and CD4⁺ cells by magnetic positive selection (Miltenyi Biotec). Cells were then sorted based on CD19⁺CD4⁺CXCR5⁺PD-1⁺ with permissive or restrictive thresholds for CXCR5 and PD-1 gating. Cells were then subjected to droplet-based single-cell RNA-seq using the Chromium Single Cell Gene Expression Solution platform (10x Genomics).
Plate-based, modified SMART-seq2 single-cell RNA-Seq. The protocol was adapted based on one described earlier (Trombetta, 2014). Target cells were directly sorted into 96-well plates with 5 μL TCL lysis buffer with 1% 2-meacaptoethanol. RNA was then captured and purified using 2.2X volume (11 μL) of RNA-SPRI beads (Beckman Coulter) followed by reverse transcription reaction as described. cDNA was then amplified, resulting in whole transcriptome amplification (WTA) products and cDNA libraries were prepared using Nextera XT DNA sample preparation reagents (Illumina) as per manufacturer’s recommendations, with some modifications. Specifically, reactions were run at ½ the recommended volume, the tagmentation step was extended to 10 minutes, and the extension time during the PCR step was increased from 30s to 60s. After the PCR step, all 96 samples were pooled without library normalization, cleaned twice with 0.9x AMPure XP SPRI beads (Beckman Coulter), and eluted in buffer TE. The pooled libraries were quantified using Quant-IT DNA High-Sensitivity Assay Kit (Invitrogen) and examined using a high sensitivity DNA chip (Agilent). Finally, samples were sequenced deeply using either a HiSeq 2000 or a HiSeq 2500 sequencer.

Data analysis for SMART-Seq 2 scRNA-Seq data. For SMART-Seq2 sequencing, RNA-seq reads were aligned to the NCBI Build 37 (UCSC mm9) of the mouse genome using TopHat (Trapnell et al., 2009). The resulting alignments were processed by Cufflinks to evaluate the abundance (using FPKM) of transcripts from RefSeq (Pruitt et al., 2007). We used log transform and quantile normalization to further normalize the expression values (TPM) within each batch of samples (i.e., all single cells in a given run). To account for low (or zero) expression values we added a value of 1 prior to log transform.

Data analysis for droplet-based 10x scRNA-Seq data. For 10x sequencing, outputs were processed with Cell Ranger (10x Genomics), and loaded into a Seurat object(286), which was
used to scale the data, regress out unwanted axes of variation (number of UMI s and ratio of
mitochondrial UMIs per library), and cluster the cells with the SLM algorithm. Default
parameter values were used unless specified otherwise. T FH signatures are manually curated and
the genes used in the signatures and Th1 signatures are based derived based on prior microarray
data (citation). Libraries were aligned with Tophat2(287), reads per transcript were counted with
featureCounts(288) and DE genes were then called with DESeq2(289) using the thresholds FDR
<= 10% and |B| >= 10% where B is the moderated B-statistic. Given a gene expression matrix A
X
m
n
for m genes over n cells, a signature is a column vector νm of length m with values of 1, -1,
and 0 corresponding to upregulated, downregulated, and non-DE genes, respectively. The
signature values for the cells are the ranked-transformed values of A Tν.

Statistical analysis for non-RNA-Seq data. Statistical analysis was performed using Prism 7
(GraphPad). Differences between two groups were compared using two-tailed unpaired T tests
and differences between two groups in multiple comparisons were done using one-way ANOVA
(n.s; * p < 0.05, ** p<0.01 and *** p<0.001). All figures show the means ± SD.

Results

Identification of Th1-like T FH cells within the T FH cell transcriptomic spectrum under NP-
OVA/CFA immunization

To characterize potential heterogeneity within a bona fide T FH pool, we performed
SMART-Seq2 single-cell RNA-seq on sorted 1,152 CD4+CD19−CXCR5−PD1+ draining lymph
node cells from wild-type mice immunized with NP-OVA/CFA for 7 days (Figure 3.1a). We
sequenced 1,152 cells and 1079 cells (~93%) passed a quality filter with 12,700 protein coding
genes detected in non-negligible levels and 4,170 genes significantly expressed based on the
expression over 4 TPM in a non-negligible group of cells. Initial clustering based on tSNE revealed an outlier cluster expressing high level of genes associated with cell cycle (Figure 3.1b). As this cluster and its genes could overshadow the biological questions we aim to pursue, the cluster was excluded from further analysis. We also excluded TFR cells based on cells that had expression positive TPM for Foxp3 or the nTreg signature (Figure 3.1c). Consequently, the data from 743 cells was acquired. We found that while no distinct clusters were apparent in t-SNE plots, these Tfh cells expressed varying degrees of Tfh signature genes (Figure 3.1d). Interestingly, Tfh cells with weak expression of Tfh signature genes had strong expression of Th1 signature genes (Figure 3.1e), suggesting that under this Th1-polarizing immunization environment, Th1-like Tfh cells were present based on transcriptomic information at the single-cell level.
Figure 3.1 Th1-like $T_{FH}$ cells are present within the $T_{FH}$ cell transcriptomic spectrum. SMART-Seq2 single-cell RNA-seq on sorted 1,152 CD4$^+$CD19$^+$CXCR5$^+$PD1$^+$ draining lymph node cells from wild-type mice immunized with NP-OVA/CFA for 7 days. Gating strategy for the sorting is shown in (a). (b) Initial tSNE plot showed an outlier cluster expressing high level of genes associated with cell cycle, which we excluded in subsequent analyses. (c) and (d) $T_{FR}$ cells identified based on cells expression positive TPM for Foxp3 or an nTreg signature were also excluded. (e) After exclusion, the resulting tSNE showed $T_{FH}$ cells with weak expression of $T_{FH}$ signature genes have strong expression of Th1 signature genes.
Figure 3.1 (continued)

a

b

Cell-cycle signature

c

d
**T-bet fate-mapping mouse model reveals the presence of T<sub>FH</sub> cells that are ex-Th<sub>1</sub> cells**

Next, we leveraged the strong Th1-polarizing environment in the NP-OVA/CFA immunization to investigate the ontogeny of the Th1-like T<sub>FH</sub> cells by crossing T-bet<sup>cre</sup> mice with Rosa26<sup>LSL-TdT</sup> mice to generate T-bet fate reporter mice. By intracellular staining and flow cytometry, we found that upon immunization, ~10% of CD4 T cells are T-bet fate-mapped (Figure 3.2a). However, when we gated on CD19<sup>+</sup>CD4<sup>+</sup>CXCR5<sup>+</sup>PD1<sup>+</sup>Foxp3<sup>+</sup>, which mark bona fide T<sub>FH</sub> cells, ~50% of them are T-bet fate<sup>+</sup> (Figure 3.2b). Among these T-bet fate<sup>+</sup> T<sub>FH</sub> cells, only ~2% of them currently express T-bet compared to ~80% seen in T-bet fate<sup>+</sup> non-T<sub>FH</sub> cells, which are likely Th1 cells (Figure 3.2c). The data suggests that a significant portion of T<sub>FH</sub> cells...
are ex-Tbet+ and possibly ex-Th1 cells, implying potential Th1/TFH linear differentiation trajectory.

**Figure 3.2** A significant portion of T<sub>FH</sub> cells from NP-OVA/CFA immunized mice previously express T-bet. T-bet fate reporter expression in total CD4 T cells is shown in (a) while T-bet fate reporter expression in T<sub>FH</sub> cells is shown in (b). T-bet expression based on intracellular staining in different populations is shown in (c).
Figure 3.3 Droplet-based single-cell RNA-seq revealed T-bet fate\(^+\) \(T_{FH}\) cells and T-bet fate\(^-\) \(T_{FH}\) cells in separate tSNE clusters. (a) tSNE plots show CD19\(^-\)CD4\(^+\)CXCR5\(^+\)PD-1\(^+\) T cells from T-bet fate reporter mice with permissive gating on CXCR5 and PD-1 and the cells are segregated as T-bet fate reporter positive (left) and T-bet fate reporter negative (right). (b) Identification of relevant clusters in the tSNE plot shows that of naïve T cells, which are T-bet fate\(^-\), Th1 cells,
which are T-bet fate+ and two of T\textsubscript{FH} clusters, one of which is T-bet fate+ and the other of which is T-bet fate-.

To confirm the presence of transcriptionally distinct T-bet fate+ T\textsubscript{FH} cells and rule out the possibility that these T-bet fate+ T\textsubscript{FH} cells only spurious express T-bet at some points in their differentiation without becoming Th1 cells, we performed 10x single-cell RNA-seq assays on CD19\textsuperscript{+}CD4\textsuperscript{+}CXCR5\textsuperscript{+}PD-1\textsuperscript{+} T cells from T-bet fate reporter mice with permissive gating on CXCR5 and PD-1 to include other cells for comparison. In this experiment, we separated cells into different lanes based on reporter expression as reporter expression at the RNA level was not reliable due to technical dropout. However, we included a lane with mixed T-bet fate+ and T-bet fate- cells to evaluate batch effects and found them to be minimal (data not shown). We identified clusters of cells including naïve T cells and Th1 cells which are T-bet fate- and T-bet fate+, respectively. Moreover, we found two clusters of T\textsubscript{FH} cells corresponding to T-bet fate reporter expression and the results showed that T-bet fate+ T\textsubscript{FH} cells, T-bet fate- T\textsubscript{FH} cells and Th1 cells formed distinct clusters in tSNE (Figure 3.3a and b), suggesting that T-bet fate+ T\textsubscript{FH} cells, T-bet fate- T\textsubscript{FH} cells and Th1 cells are transcriptionally distinct at the single-cell level.

Consistent with what we observed in flow cytometry results, T-bet fate+ T\textsubscript{FH} cells minimally express T-bet in single-cell RNA-seq profiles (Figure 3.4a). However, they still retained expression of Th1 signature genes, including Ifng, Cxcr3 and Stat4 (Figure 3.4b to d), suggesting that these T\textsubscript{FH} cells had not just turned on T-bet expression spuriously, but that the Th1 gene expression program was turned on together with T-bet in these cells.
**Figure 3.4** Droplet-based single-cell RNA-seq showed that while T-bet fate\(^ {+} \) T\(_{FH} \) cells mostly extinguish T-bet expression, the cells still express residual differential expression of Th1 cell-associated genes. Th1-associated gene expression is shown in tSNE plots with the relevant clusters identified in **Figure 3.3b**. The genes include T-bet-encoded Tbx21 (a), aggregated Th1 signature genes (b), Ifng (c), Cxcr3 (d) and Stat4 (e).
Figure 3.4 (continued)

a

Tbx21 (encoding T-bet)

b

Th1 signature genes
Figure 3.4 (continued)
T-bet fate$^+$ T$_{FH}$ cells are transcriptionally and functionally distinct from T-bet fate$^-$ T$_{FH}$ cells.

To assess if T-bet fate$^+$ T$_{FH}$ cells are transcriptionally distinct from T-bet fate$^-$ T$_{FH}$ cells, we performed SMART-Seq2 single-cell RNA-seq on 1,152 CD4$^+$CD19$^+$CXCR5$^+$PD1$^+$ cells with restrictive gating from T-bet fate reporter mice to ensure that only T$_{FH}$ cells were included in this experiment (Figure 3.5a). After QC and filtering with the same strategy done earlier, 662 cells T$_{FH}$ cells remained. In this experiment, T-bet fate$^+$ T$_{FH}$ cells existed but did not form a distinct cluster from T-bet fate$^-$ T$_{FH}$ cells (Figure 3.5b). This result supports that the cells are likely to derive from Th1 rather than other T$_{FH}$ cells. Furthermore, even if T-bet fate$^+$ T$_{FH}$ cells did not form a distinct cluster in this experiment, they possessed a spatial feature associated with PC2, the dimension that corresponds to Th1 signature strength similar to what was observed earlier (Figure 3.1c) as their frequency of T-bet fate$^+$ T$_{FH}$ cells increased linearly along the axis, supporting the relationship of T-bet fate$^+$ T$_{FH}$ cells with Th1 cells based on shared gene expression (Figure 3.5c). We also found that when compared to T-bet fate$^-$ T$_{FH}$ cells, T-bet fate$^+$ T$_{FH}$ cells have more activated phenotype with significant positive correlation to Th1 signature genes but negative correlation to T$_{FH}$ genes, except for Stat3 and Hif1a (Figure 3.5d).
Figure 3.5 T-bet fate$^+$ $T_{FH}$ cells are transcriptionally and functionally distinct from T-bet fate$^-$ $T_{FH}$ cells. SMART-Seq2 single-cell RNA-seq on sorted T-bet fate$^+$ $T_{FH}$ cells and T-bet fate$^-$ $T_{FH}$ cells (defined as CD4$^+$CD19$^+$CXCR5$^+$PD1$^+$) from draining lymph node cells of T-bet fate reporter mice immunized with NP-OVA/CFA for 7 days. Sorting strategy is shown in (a). (b) tSNE plot shows that T-bet fate$^+$ $T_{FH}$ cells and T-bet fate$^-$ $T_{FH}$ cells did not form distinct clusters in this experiment. (c) Cells were computationally sorted according to PC2 and the ratio of T-bet fate$^+$ cells out of all $T_{FH}$ cells analyzed was plotted in equally-sized bins with the x-axis corresponding to an index that reflects the distance in the aforementioned PC2 and the y-axis corresponding to the aggregated ratio of the T-bet fate$^+$ $T_{FH}$ cells. (d) Differentially-expressed genes and signatures between T-bet fate$^+$ $T_{FH}$ cells and T-bet fate$^-$ $T_{FH}$ cells are shown with the fold difference corresponding to the expression in T-bet fate$^+$ $T_{FH}$ cells over T-bet fate$^-$ $T_{FH}$ cells (i.e. the positive values mean the expression is more enriched in T-bet fate$^+$ $T_{FH}$ cells and the negative values mean the expression is more enriched in T-bet fate$^-$ $T_{FH}$ cells. Statistical significance is shown as adjusted p values.
Figure 3.5 (continued)
Figure 3.5 (continued)

d

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STAT3 is a mediator of T-bet fate+ T_{FH} cells as it drives Th1/T_{FH} conversion.

Because STAT3 is a positive regulator of T_{FH} cells and negative regulator of Th1 cells(301), we hypothesize that Th1 cells could be driven to become Th1-like, T-bet fate+ T_{FH} cells by overexpressing STAT3. To investigate if STAT3 is a mediator of T-bet fate+ T_{FH} cells, we crossed T-bet fate reporter mice with STAT3^{fl/fl} mice, so that STAT3 would be deleted upon T-bet expression. Based on flow cytometry, total T-bet fate+ CD4 T cells were similar between WT and the STAT3 conditional knockout mice (Figure 3.6a). Strikingly, however, T_{FH} cells were significantly reduced in the STAT3 conditional knockout mice and the remaining T_{FH} cells are all T-bet fate (Figure 3.6b). The reduction was only seen in T-bet fate population, but not in the T-bet fate population as there were almost no T-bet fate T_{FH} cells in the STAT3 conditional knockout mice (Figure 3.6c and d). The results demonstrate that induction of STAT-3 expression in Th1 cells mediated the transition of Th1 cells to T_{FH} cells in vivo.
Figure 3.6 STAT3 is a mediator of T-bet fate\(^+\) \(T_{FH}\) cells as it drives Th1/\(T_{FH}\) conversion. Cells from either T-bet fate reporter mice or T-bet fate reporter mice (designated as WT) crossed to STAT3\(^{+/0}\) mice, which delete STAT3 in T-bet\(^+\) cells (designated as STAT3 CKO), were analyzed by flow cytometry and cellular compositions in the mice are specified by each panel.
Figure 3.6 (continued)

(a) Gated on CD19^CD4^Foxp3^- WT vs. STAT3 CKO

(b) Gated on CD19^CD4^Foxp3^- WT vs. STAT3 CKO
Discussion

Using single-cell RNA-seq experiments, we demonstrated a degree of cellular heterogeneity of draining lymph node T_{FH} cells from mice immunized with NP-OVA/CFA as seen by T_{FH} cells with weaker expression of T_{FH} signature genes and stronger expression of Th1 signature genes. Combining these experiments with the use of a T-bet fate-mapping mouse strain, we found a significant portion of T_{FH} cells that previously expressed T-bet and still
expressed residual Th1 cell-associated genes, supporting a linear differentiation trajectory in which these T<sub>FH</sub> cells are derived from Th1 cells. Based on gene expression analysis, we predicted STAT3 as a mediator of Th1/T<sub>FH</sub> cell transition and found that deletion of STAT3 in T-bet<sup>+</sup> cells resulted in severe defects in those T-bet fate<sup>+</sup> T<sub>FH</sub> cells. Taken together, the results support the linear differentiation trajectory of these Th1-like T<sub>FH</sub> cells even though we cannot rule out the possibility of a co-existing lineage differentiation model as our experiments did not address the ontogeny of the remaining T-bet fate<sup>-</sup> T<sub>FH</sub> cells in the same immunization settings. Moreover, whether such linear differentiation trajectory exists in other helper T cells in different immunization and infection contexts has yet to be investigated.

Previous results showed the relationship between T-bet, Bcl-6 and T<sub>FH</sub> cells (302-305) as T-bet was shown to be important for IFN-γ production in T<sub>FH</sub> cells, which is likely to be important for biasing isotype switching to IgG2a. Thus, it is not surprising that co-ordination of cell-mediated immune responses and humoral immune responses could be accomplished through shared Th1/T<sub>FH</sub> cell ontogeny. For example, early Th1 cell differentiation was shown to be marked by a T<sub>FH</sub> cell-like transition (306). In addition, T-bet and Bcl-6 can be co-expressed in T<sub>FH</sub> cells at early stage of infection (181, 306-309). A recent study using a T-bet fate-mapping mouse strain also demonstrated results consistent to our work as they found that transient T-bet expression functionally specified Th1-like T<sub>FH</sub> cells even though they did not directly address the ontogenic question (310).

A key piece of evidence in our work that supports the linear model is the perturbation of STAT3 in T-bet fate<sup>+</sup> T<sub>FH</sub> cells resulted in striking loss of Th1-like, T-bet fate<sup>+</sup> T<sub>FH</sub> cells. Such strong phenotype was unexpected as prior results in CD4 cell-specific deletion of STAT3 showed more nuanced T<sub>FH</sub> cell phenotype. In one study, deletion of STAT3 in CD4 cells using
STAT3\(^{ \text{+/+} }\) x CD4\(^{ \text{cre} }\) mice, showed no difference in the frequency of T\(_{\text{FH}}\) cells unless STAT1 expression was also inhibited (311), implying a potential interaction between STAT3 and STAT1 in T\(_{\text{FH}}\) cell differentiation. As STAT1 is an important transcription factor in Th1 cell differentiation and induction of T-bet, these findings also support the relationship between Th1 cells and Th1-like T\(_{\text{FH}}\) cells seen in our work. Another study using STAT3\(^{ \text{+/+} }\) x CD4\(^{ \text{cre} }\) mice, however, showed that T\(_{\text{FH}}\) cells in the STAT3 knockout, though not different in their abundance, were transcriptionally more similar to Th1 cells (312). Considering the findings in our study, the phenotype was unexpected as those Th1-like T\(_{\text{FH}}\) cells should be deficient in the absence of STAT3. One potential explanation is that in the absence of STAT3 in all CD4 T cells, ex-Th1 T\(_{\text{FH}}\) cells are also absent while non ex-Th1 T\(_{\text{FH}}\) cells under Th1-polarizing conditions compensate by adopting Th1-like features. In fact, Th1 polarizing pressure may be implicated in the context of influenza infection in which protective IgG2 neutralizing antibody responses were still intact in the absence of T\(_{\text{FH}}\) cells in Bcl6-deficient mice as Th1 cells adopted some T\(_{\text{FH}}\) cell aspects like the production of IL-21 without the capacity to enter GCs (145). The nuanced phenotype regarding STAT3 and T\(_{\text{FH}}\) cells was also seen in Job's syndrome patients with STAT3 loss-of-function mutations as circulating CXCR5\(^{ \text{CD4}^{+} }\) T cells were only partially deficient in their abundance while IgE levels were elevated (313). Although there are phenotypic discrepancies among studies of STAT3 deficiency in different contexts, our novel key findings relating to the role of STAT3 in Th1-like T\(_{\text{FH}}\) cells were only revealed with T-bet\(^{+}\) cell-specific STAT3 deletion.

Differentiation of Th1 cells to Th1-like T\(_{\text{FH}}\) cells is potentially driven by the inflammatory milieu that promote STAT3-mediated T\(_{\text{FH}}\) cell induction due to certain cytokines. IL-6, IL-21 and IL-27, all of which signal through STAT3, have been implicated in T\(_{\text{FH}}\) cell
development, albeit with different roles. IL-6 was shown to be important for early development of T\textsubscript{FH} cells following viral challenge (311). It was also implicated in their maintenance later in chronic viral infection (314) while IL-27 was shown to be important for the maintenance upon protein immunization (315). IL-21, moreover, has been reported to be important for T\textsubscript{FH} cell differentiation (31, 316) although such a role has not been universally established with discrepancies seen in different immunization contexts (48, 49). In the absence of IL-6, IL-21 is more important in later stages upon viral infection and protein immunization (317, 318) even though it is not required in early T\textsubscript{FH} cell differentiation (311). Deleting receptors for such cytokines in T-bet\textsuperscript{+} cells by crossing mice harboring flox alleles in corresponding cytokine receptor loci with T-bet fate-mapping mice could address the potential roles of cytokines upstream of STAT3 that drives Th1/T\textsubscript{FH} cell transition.

Taken together, we uncovered a linear differentiation trajectory of T\textsubscript{FH} cells from Th1 cells in which Th1 cells extinguish expression of T-bet and adopt T\textsubscript{FH} cell phenotype. The acquisition of T\textsubscript{FH} cell effector gene modules, which represent a “follicular” phenotype, after Th1 cell commitment implies the presence of effector “follicular” gene modules that other helper T cells could adopt and add to their existing helper T cells gene program. Dissecting mechanisms underlying such adoption of effector gene modules beyond existing “basal” Th cell differentiation could provide novel targets for modulating context-specific antibody responses for therapeutic application.
Chapter 4

General Discussion
Molecular interaction among $T_{FH}$ cells, $T_{FR}$ cells and B cells in the GCs

As $T_{FH}$ cell help is a key factor in regulating multiple processes in the GCs, the existence of $T_{FR}$ cells adds another cellular checkpoint that influences those processes. $T_{FH}$ cell function must be tightly regulated to ensure optimal GC responses and such regulation can be different from that of other CD4 helper T cells. For example, excessive $T_{FH}$ cell help does not always translate into elevated B cell help, as high numbers of $T_{FH}$ cells can limit affinity maturation due to those oversaturated $T_{FH}$ cells being less discriminating at B cell selection (319). Moreover, the balance of GC reaction outputs, either as plasma cells, memory B cells or recycling GC B cells, is contingent on the level of $T_{FH}$ cell help (320). CSRs are also driven by cytokines produced by $T_{FH}$ cells and the capacity of $T_{FH}$ cells to orchestrate different antibody isotypes suggests their important roles in this aspect as described earlier. Thus, both number and function of $T_{FH}$ cells must be tightly regulated to allow for multiple outcomes in B cell function.

Our work has provided more insight into $T_{FH}$ cell regulation, both through $T_{FH}$ cell-intrinsic and $T_{FH}$ cell-extrinsic mechanisms. For example, the capacity for Th1 cells to be redirected to a $T_{FH}$ cell phenotype in a linear fashion is likely to ensure coordinated type 1-polarized microenvironments for humoral responses. One subsequent implication is that because $T_{FH}$ cells would have a large transcriptional and epigenetic footprint of Th1 lineage-specific transcription factors with limited accommodation of the $T_{FH}$ related gene program in the linear model we demonstrated, the relative importance of the $T_{FH}$ related gene program should be revisited in a more contextualized fashion. In fact, it has been shown that in mice that lack $T_{FH}$ cells and germinal centers, immunization with inactivated influenza A virus still resulted in protective IgG2 responses against highly virulent influenza A infection even though the IgG2 antiviral antibodies were of low affinity and the IgG1 responses were mostly abolished (145).
This implies that regulation of different antibody isotypes can be highly compartmentalized, with differential dependence on different Th1/ThFH related gene programs. Another implication is that as the ThFH master transcription factor Bcl6 is likely to be expressed in a Th1-specific epigenetic landscape in the linear model, its downstream ThFH-like functional states can be speculated to require less genetic and epigenetic commitment. Thus, reversibility of the ThFH cell phenotype should be easier, suggesting that ThFH cell effector gene modules, which represent their "follicularity", might confer phenotypic diversification at the level of Th1 functional states although epigenetic experiments have yet to be done to verify this.

On the other hand, the presence of TFR cells provides ThFH cell-extrinsic regulation, we demonstrated in Chapter 2 that Fgl2 is a TFR cell effector molecule that shapes ThFH cell-mediated cytokine microenvironments, and subsequently suppresses type-2 CSR. While the results appear to be unexpected, as it was shown previously that TIGIT+, Fgl2-producing Treg cells selectively suppress Th1 cells and Th17 cells but not Th2 cells (117), we also observed Fgl2 functions to be context-dependent with respect to the presence of B cells. However, because our immunization strategy (NP-OVA/CFA) was type-1-biased, the suppression of type-2 antibody responses mediated by Fgl2-producing TFR cells might account for the responses appropriate for the type-1 inducing microenvironments. The selective influence of this TFR cell effector molecule on IgG1 was also consistent with its relative dependence on ThFH cells and germinal centers compared to IgG2 in influenza infection (145). Future studies need to explore whether different immunization strategies and/or different types of infection can affect Fgl2 production by TFR cells and subsequent antibody isotype responses, although there has been some evidence showing that IFN-γ induces Fgl2 production in macrophages (229).
TFR cell functions appear to be predominantly associated with the size of TFH cell and GC B cell populations (146-148). Our data showing that Fgl2 only suppresses B cell number in vitro in case of either B cells being activated through BCRs and/or B cells lacking cognate TFH cell help is consistent with the earlier findings showing that TFR cells restrict the outgrowth of non-antigen-specific B cells clones in the germinal center (147). The fact that Fgl2 has no effect on B cell number in the presence of TFH cells in vitro suggested that TFH cell help can provide B cells with resistance against TFR cell-mediated suppression.

The role of TFR cells in regulating antibody affinity has not yet been fully elucidated. TFR cells have been implicated in suppressing both low- and high-affinity antibody production (146). In adoptive transfer studies, recipients of CTLA-4-deficient TFR cells, which were shown to be functionally deficient, had increased serum Ag-specific antibody that tended to be of lower affinity (152) although better assessments of affinity maturation are still required for more definitive conclusions. These results, nevertheless, suggest that TFR cells may not only suppress antibody responses overall, but also may facilitate the generation of the high affinity B cell clones, either through direct B cell inhibition or through regulation of TFH cells. The role of TFR cells in this aspect, therefore, would have a paradoxical role in modulating antibody responses as they limit overall antibody production but also promote the selection for the high-affinity clones, similar to the death receptor CD95/FAS (321). This concept has important implications. For example, in the context of vaccination, inhibiting TFR cells may result in more total antigen-specific antibodies but at the same time the antibodies might have poorer affinity. Conversely, enhancing TFR cell suppressive function could result in selection of the highest affinity clones, which may provide better neutralizing activity, and in selection of higher affinity plasma cells and/or memory B cells.
With respect to memory B cell generation, the canonical model suggests that low affinity B cells receiving low T<sub>FH</sub> cell help favor B cells exiting GCs as memory B cells as described earlier. Thus, T<sub>FR</sub> cells should promote memory B cell generation. However, there is insufficient evidence to support this role for T<sub>FR</sub> cells, and our data on Fgl2-deficient mice showed no effects on the memory B cell population upon immunization (data not shown). A more recent discovery of IL-9-producing T<sub>FH</sub> cells that favor memory B cell generation (211) suggests that the degree of T<sub>FH</sub> cell help alone does not completely account for GC B cell fates, as the quality of help also appears to be important. Whether IL-9-producing T<sub>FH</sub> cells are a distinct T<sub>FH</sub> cell subset remains to be further investigated, although we have not found such evidence in our single-cell RNA-seq datasets. However, because the frequency of IL-9-producing T<sub>FH</sub> cells was shown to be low (~2%), an analysis with a larger number of cells might be required to address this question. If such specialized T<sub>FH</sub> cells favoring memory B cell generation exist, we speculate that their TCR affinity should be relatively higher as they are capable of helping B cells with low affinity. Interestingly, it has been shown that efficient affinity maturation occurs in the absence of homogenizing selection such that many B cell clones were able to mature in parallel within the same GCs (322), raising the possibility that different T<sub>FH</sub> with variable TCR affinities can be present in the same GCs as well. Thus, integrating single-cell RNA-seq information with single-cell TCR sequencing with the advent of a more recent advance in single-cell genomic technology would allow us to explore such a possibility. Another potential mechanism regarding memory B cell generation is that as IL-9 was shown to promote Treg function (213), the effects of those IL-9-producing T<sub>FH</sub> cells could be through mediation of T<sub>FR</sub> function, which then limit T<sub>FH</sub> cell help. If true, this would also underline an intricate relationship with multiple feedback mechanisms among T<sub>FH</sub> cells, T<sub>FR</sub> cells and GC B cells to reach appropriate effector B cell outputs.
If such differential regulation of $T_{FR}$ cell effector function exists, the heterogeneity of $T_{FR}$ cells should also be explored more closely. Single-cell RNA-seq experiments have been performed on splenic conventional T cells ($T_{conv}$ cells; defined as $CD4^+TCR\beta^+Foxp3^+$) and Treg cells (defined as $CD4^+TCR\beta^+Foxp3^+$) from Foxp3$^{EFP}$ reporter mice. The results, based on unsupervised clustering in a tSNE plot, revealed a cluster of cells enriched in $T_{FH}/T_{FR}$ cell-associated genes and composed of both $T_{conv}$ and Treg cells, implying that potential $T_{FH}$ cells and $T_{FR}$ cells, while having markedly opposite functions, might possess related transcriptional profiles that are overlapping despite the clear distinction observed with Foxp3 reporter expression (156). The study, however, only identified cells based on $T_{FH}/T_{FR}$ signature genes, not by protein markers (CXCR5 with PD-1 or ICOS) for functional validation. On the other hand, our single-cell RNA-seq data performed on permissively-gated $CD4^+CXCR5^+PD-1^+$ lymph node cells upon NP-OVA/CFA immunization, a population expected to be enriched in both bona fide $T_{FH}$ cells and $T_{FR}$ cells, provided a different picture. In our case, $T_{FR}$ cells appeared to be more similar to effector Treg cells as they were in the same cluster in a tSNE plot upon unsupervised clustering, while $T_{FH}$ cells were in their own, distinct clusters. Our experiment, however, did not take the identity of Treg cells based on the bona fide definition with respect to dichotomous Foxp3 reporter expression into account before cell sorting. Such inconsistencies among two single-cell RNA-seq experiments, therefore, demonstrate that how cells are defined and selected to be subjected to such experiments could affect the results and the subsequent interpretation. To resolve the issue as complex as $T_{FR}$ cell biology with significant overlaps with other different T cells, experimental designs that carefully integrate well-defined, functionally-validated cell identities in addition to unbiased clustering of heterogeneous cell pools will be required. Just as in the case of $T_{FH}$ cells, questions regarding the “follicularity” as a distinct lineage or it only
represents a functional state that might even be functionally malleable should be addressed with the experiments similar to those done in T_{FH} cells with the potential integration with epigenetic information.

**T_{FR} cells as potential regulators of autoreactive B cells**

Pioneering work on T_{FR} cells focused on their regulatory effects on antigen-specific B cells and their effector functions were shown to be the direct opposite of those in T_{FH} cells. However, growing evidence has suggested that T_{FR} cells might be more important in suppressing autoreactive B cells spuriously activated during inflammation. With exceptions in some specific contexts (155), T_{FR} cells were shown to generally come from naturally-occurring thymically-derived Treg cells (146-148) and their TCRs are more similar to Treg cells than T_{FH} cells (154). As thymically-derived Treg cells harbor TCRs specific for self-antigens, T_{FR} cells might potentially provide direct cognate suppression on autoreactive B cells as those B cells present autoantigens. Our findings on Fgl2 also reveal that T_{FH} cell help can overcome T_{FR} cell cytotoxic effects on B cells that receive cognate help from T_{FH} cells, whereas autoreactive B cells receive no such help and thus those autoreactive B cells are probably susceptible to elimination. The phenotypes observed in T_{FR} cell-deficient mice also support the dominant role of T_{FR} cells as regulators of autoreactive B cells. In those mice, it was shown that T_{FR} cells did not significantly affect the influenza-specific B-cell responses upon infection, but instead promoted the accumulation of ANA ASCs (83). In another independent study, T_{FR} cell-deficient mice developed late-onset spontaneous autoimmunity affecting salivary glands with lymphocyte infiltration and antibody deposition and the mice had more severe disease in a mouse experimental Sjögren’s syndrome model (150). The breakdown of tolerance, either
spontaneously in older mice or upon inflammation, was also consistent with the phenotypes observed in Fgl2-deficient mice, although the phenotypes in the latter case appear to be more severe, suggesting that there could be additional sources of Fgl2 beyond T_{FR} cells that might also play a role in regulating autoimmunity and tissue inflammation. Nonetheless, these findings suggest that autoreactive B cells could get activated upon inflammation and, without functional T_{FR} cells, those B cells could lead to development of autoimmunity, a phenomenon that appears to be accumulative such that the phenotypes, which are variable in the case of Fgl2-deficient mice, might readily be seen in older mice that experience countless yet variable inflammatory events throughout their lifespans. Taken together, we summarize potential mechanisms of T_{FR} cells with respect to T_{FH} cells, antigen-specific B cells and autoreactive B cells in Figure 4.1.

**Figure 4.1** T_{FR} cell effector functions. T_{FR} cells can inhibit antibody responses by directly regulating T_{FH} cells and antigen-specific B cells. T_{FH} cells can both promote antigen-specific B cells and counteract T_{FR} cell-mediated suppression by conferring resistance to B cells against T_{FR}
cell effector responses. $T_{FR}$ cells, which harbor TCRs that are likely to recognize self-antigens, might directly suppress autoreactive B cells through cognate interaction.

Different self-tolerance mechanisms have been shown to limit autoreactive B cells at different B cell stages and locations, including clonal deletion, receptor editing, anergy induction and clonal ignorance (323). In autoimmune-prone mouse models, autoreactive B cells get activated, although the requirement for GCs appears to be context dependent. Extrafollicular sites, such as the T cell zone/red pulp border in the spleen, were shown to play dominant roles in some contexts, including responses for autoreactive specificities such as RF and anti-DNA in MRL/llpr, NZB/W and RF mouse models (324-326). Interestingly, the resulting B cells secreting autoantibodies were shown to undergo many somatic hypermutations, affinity maturation, clonal expansion and CSRs independent of GCs (327). In a transgenic mouse model of B cells harboring BCRs against an autoantigen Smith (Sm) ribonucleoprotein, anti-Sm B cells comprise 30%-50% of peripheral B cells and are present as transitional, follicular and marginal zone B cells in the spleen and as B-1 cells in the peritoneum (328, 329). However, there was no significant difference in serum anti-Sm antibodies between the transgenic mice and non-transgenic mice, indicating the presence of a checkpoint at plasma cell differentiation (328). In fact, those anti-Sm B cells were shown to be present at the pre-plasma cell stage with intermediate expression of CD138 in B cell follicles close to the bridging channels and T cell zones in the spleen of non-transgenic mice and MRL/llpr mice at steady state. However, these B cells fail to up-regulate Prdm1 to fully become antibody-secreting plasma cells (330). While T cell-independent B cell responses are known to occur in extrafollicular sites (331), the involvement of T cells, especially $T_{FH}$ cells and $T_{FR}$ cells, in terms of their influence on

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autoreactive B cells in those sites, cannot be entirely excluded as the cells have been associated with multiple autoimmune diseases (332, 333). Such studies suggest the presence of a regulatory checkpoint after initial B cell activation and it would be interesting to see if T_{FR} cells, or even non-T_{FR} Treg cells, could account for that checkpoint. In fact, in one of our adoptive transfer experiments, anti-dsDNA ASCs were transiently up-regulated in CD28-deficient mice, which lack GCs. However, those autoreactive B cells could be suppressed upon adoptive transfer of T_{FR} cells in an Fgl2-dependent fashion. The results suggest that induction of autoreactive B cells in CD28-deficient mice is likely to occur outside GCs, but at what sites and stages those autoreactive B cells got activated and suppressed upon T_{FR} cell transfer remains to be further investigated.

Autoreactive B cells are likely to be at a disadvantage at entering GCs as adoptive transfer of autoreactive B cells expressing the anti-apoptotic Bcl2 transgene were shown to be short-lived and excluded from B cell follicles in a transgenic mouse model (334). Moreover, these cells are more difficult to be activated than non-autoreactive B cells (335). Thus, it is likely that in order to enter GCs, these autoreactive B cells require BCR crosslinking by high-affinity multivalent autoantigens and/or co-stimulatory signals like TLRs in order to compete with non-autoreactive B cells to enter GCs. Despite such disadvantages, however, autoreactive B cells have been shown to be able to enter GCs and dysregulated T_{FH} cells have been shown to be important in supporting those autoreactive B cells. For example, in the sanroque lupus model in which the ICOS pathway was dysregulated (336), it has been shown that T_{FH} cells are required for the systemic autoimmunity (337), which is associated with decreased Ifng mRNA decay causing excessive IFN-γ signaling in T cells, resulting in accumulation of T_{FH} cells, spontaneous GC, autoantibody formation, and glomerulonephritis (300). Autoreactive B cells in GCs, on the
other hand, have been shown to be restrained through multiple mechanisms, including deletion by FDCs displaying self-antigens (338) and selection of mutated autoreactive clones away from autoreactivity (339, 340). A more recent work has shown that the redemption of such autoreactive B cells could even confer an increase in affinity against cross-reactive foreign antigens with 5,000-fold preferential binding to foreign over self-epitopes (341). The capacity of autoreactive B cells to be redeemed in the GCs could be important in cases where pathogens mimic or shield themselves with host antigens. Broadly neutralizing antibodies against HIV-1, for example, have been shown to cross-react with self-antigens (342) and poly-reactivity was found in 75% of a large collection of human monoclonal antibodies against HIV (343). Interestingly, knock-in experiment of B cells expressing germline alleles encoding broadly neutralizing antibodies against HIV-1 frequently showed clonal deletion or absence of allelic exclusion, which is indicative of self-reactivity (344, 345). Such findings imply that B cells producing those broadly neutralizing antibodies might potentially come from autoreactive precursors. Somatic hypermutation in GCs can also result in generation of autoreactive B cells (346, 347) and many autoimmune mouse models have been shown to have spontaneous GC formation (348). A recent study demonstrates that a single autoreactive BCR transgenic B cell clone was able to drive spontaneous GC formation with expansion of other autoreactive B cells derived from wild-type B cells via epitope spreading, while blocking CD40L significantly ablated such GCs, suggesting the involvement of T<sub>FH</sub> cell help (349). While T<sub>FR</sub> cells are expected to be able to recognize anergic B cells, which are exposed as autoreactive, whether T<sub>FR</sub> cells are programmed to recognized clonally ignorant B cells, which behave more like non-autoreactive B cells that are sequestered from the immune system, remains to be further explored as the role of T<sub>FR</sub> cells in controlling epitope spreading is unknown. Taken together, the tension
of eliminating autoreactive B cells in GCs to prevent autoimmunity and selection of non-autoreactive and/or mutated, redeemed B cells that recognize foreign antigens, therefore, might involve active competition between $T_{FR}$ cells and $T_{FH}$ cells.

$T_{FH}$ cells and $T_{FR}$ cells also potentially play important roles in tertiary lymphoid structures (TLS), which are microanatomical structures sharing features with secondary lymphoid organs. Those features include segregated T and B cell clusters, presence of FDC networks, high endothelial venules and specialized fibroblasts (350). TLS support recirculation of T and B cells through expression of chemokines including CXCL13, CCL21 and CCL19, as well as peripheral lymph node addressin (PNAd) (351-353). However, TLS often lack the complex anatomical compartmentalization seen in secondary lymphoid organs and the formation of functional GCs is highly variable within and among diseases (350). TLS are able to promote adaptive immune responses against local antigens and are usually associated with poor prognosis of chronic autoimmune diseases, which could be attributed to their relatively disorganized structures, resulting in the absence of appropriate checkpoints for autoreactive cell screening (350).

Involvement of T cell help and Treg-mediated suppression in TLS has been shown in the context of atherosclerotic plaque as ICOS-L blockade reduced TLS and aberrant plaque formation while depletion of Treg cells had the reverse effects (354, 355). Th17 cells were shown to adopt $T_{FH}$-like phenotype and mediate TLS in EAE in a manner that is partially dependent on IL-17 and podoplanin (356). In RA patients, PD1$^{hi}$CXCR5 CD4$^+$ T cells in synovium termed “peripheral helpers” were shown to possess some $T_{FH}$-like phenotype and promote antibody production (357). Expansion of $T_{FH}$ cells in salivary glands was reported in primary Sjögren’s Syndrome (358, 359). While the involvement of $T_{FR}$ cells in TLS was never

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fully elucidated, the findings that $T_{FR}$ cell-deficient mice developed autoimmune phenotype in salivary glands spontaneously and upon induction in an experimental Sjögren’s Syndrome model suggest the potential involvement of $T_{FR}$ cells in controlling TLS (150). Interestingly, the TLS T cells that mediate antibody responses in the periphery in the cases mentioned above appear to possess only partial $T_{FH}$ gene program with different phenotypes in different diseases. In addition, the ontogenic nature of these $T_{FH}$-like TLS T cells has still yet to be fully investigated. In a similar line of questioning we had in our single-cell RNA-seq experiments, it would be interesting to see whether the $T_{FH}$-like TLS T cells follow a linear model as in the case of EAE where Th17 cells can adopt some $T_{FH}$-like phenotype or it is a lineage model where $T_{FH}$ cells downsize their gene programs as seen in circulating “memory” $T_{FH}$ cells in which they extinguish Bcl-6 expression after leaving GCs (181, 360, 361) and potentially up-regulate effector genes corresponding to peripheral microenvironments.

Co-inhibitory molecules on follicular T cells

Our results demonstrated that Fgl2 was able to induce expression of co-inhibitory molecules, including PD-1, TIGIT, LAG-3 and TIM-3. However, knowledge on the roles of these co-inhibitory molecules in $T_{FH}$ cells and $T_{FR}$ cells is still limited. PD-1 is highly expressed both in $T_{FH}$ cells and $T_{FR}$ cells and was shown to limit $T_{FR}$ cell number and functions (279). In $T_{FH}$ cells, PD-1 controls $T_{FH}$ cell positioning and functions within GCs as PD-1 inhibits $T_{FH}$ cell recruitment into the follicle and also restrict CXCR3 upregulation (362). We also observed high expression of TIGIT both in $T_{FH}$ cells and $T_{FR}$ cells while expression of LAG-3 is intermediate and expression of TIM-3 is low in $T_{FH}$ cells (data not shown). The role of TIM-3 in $T_{FH}$ cells has been overlooked, probably due to its low expression in $T_{FH}$ cells. However, among the co-
inhibitory molecules up-regulated by Fgl2, the change in TIM-3 at the protein level in T<sub>FH</sub> cells was the most dramatic. Notably, germline HAVCR2 (encoding TIM-3) mutations were shown to be associated with subcutaneous panniculitis-like T cell lymphomas with some patients exhibiting increased IgE levels and positive anti-DNA antibodies (363), the phenotypes significantly overlapped with the spontaneous diseases observed in aged Fgl2-deficient mice. The observations imply potential involvement of T<sub>FR</sub> cells regulating T<sub>FH</sub> cells through the modulation of TIM-3 expression in the latter although future work needs to be done to confirm this mechanism.

**T<sub>FH</sub>-like CD8 T cells**

More recent data has suggested the presence of T<sub>FH</sub>-like CD8 T cells with divergent effector functions beyond the support of antibody responses. CXCR5<sup>+</sup> CD8 T cells possessing gene expression profiles resembling those of CD4 T<sub>FH</sub> cells were shown to harbor enhanced cytotoxicity and anti-viral functions (364, 365). The phenotypic overlap was later shown in CD8 T cells in the context of T cell exhaustion, which is dependent on expression of co-inhibitory molecules. Accumulated expression of co-inhibitory molecules has been suggested to mark hierarchical stages of T cell exhaustion (366) and PD-1<sup>+</sup> TIM-3<sup>+</sup> CD8 T cells were shown to be more exhausted compared to PD-1<sup>+</sup> TIM-3<sup>+</sup> CD8 T cells (partially exhausted) and PD-1<sup>+</sup> TIM3<sup>-</sup> CD8 T cells (most functional) in cancer (367). More recently, it was shown that upon chronic LCMV infection, CXCR5<sup>+</sup>PD1<sup>+</sup> TIM-3<sup>-</sup> T<sub>FH</sub>-like CD8 T cells act as stem cells that can differentiate into more exhausted CXCR5<sup>-</sup>PD1<sup>+</sup> TIM-3<sup>+</sup> CD8 T cells. After PD-1 blockade, a CD8 proliferative burst almost exclusively comes from these CXCR5<sup>+</sup>PD1<sup>+</sup> T<sub>FH</sub>-like CD8 T cells (368). Additionally, PD-1 blockade was shown to be dependent on CD28 and PD1<sup>+</sup> TIM-3<sup>-</sup> CD8
T cells, which are possibly the $\mathbf{T_{FH}}$-like ones, and express a significantly higher level of CD28 than PD1$^+$TIM-3$^+$ cells (369, 370). Thus, there seems to be a level of antagonism between expression of TIM-3 and stem-like features possessed by CXCR5$^+$PD-1$^+$ $\mathbf{T_{FH}}$-like CD8 T cells.

In addition, we might be able to revisit what it means to harbor $\mathbf{T_{FH}}$-like features. In chronic LCMV infection, CXCR5$^+$PD-1$^+$ $\mathbf{T_{FH}}$-like CD8 T cells act as stem cells as they undergo a slow self-renewal and are able to quickly proliferate upon activation to give rise to the more terminally differentiated effector-like CD8$^+$ T cell subset that is present in both lymphoid and non-lymphoid tissues (368). Whether such stemness with renewal capacity exists in CD4 $\mathbf{T_{FH}}$ cells, or even TFR cells, and whether it is equivalent to that found in memory T cells still require further investigation. However, one potential mechanism might involve the role of a transcription factor TCF-1 (encoded by the gene $\mathit{Tcf7}$), which was shown to play a role in the maintenance of haematopoietic stem cells in an undifferentiated state (371) and this transcription factor is shown to be required for both CD4 $\mathbf{T_{FH}}$ differentiation (372, 373) and the generation of CXCR5$^+$PD-1$^+$ $\mathbf{T_{FH}}$-like CD8 T cells (368). Integrating the existing knowledge based on gene expression profiling and single-cell gene expression profiling might allow us to dissect the heterogeneity within $\mathbf{T_{FH}}$ cells, as exemplified in our work, and CD8 T cells and identify shared gene regulatory modules that could shed new light on the biology of both $\mathbf{T_{FH}}$ cells and $\mathbf{T_{FH}}$-like CD8 T cells.

Such phenotypic overlap in CD4 $\mathbf{T_{FH}}$ cells and $\mathbf{T_{FH}}$-like CD8 T cells, which are two immune cells with very different effector functions, begs the questions regarding the underlying biological aspects of the $\mathbf{T_{FH}}$ cell gene program that might have been underappreciated. Consequently, it might be possible to exploit the knowledge regarding the two cell types to generate new hypotheses about the biology of each one. For example, while strong antigen/TCR
binding was shown to favor T\textsubscript{FH} cell differentiation over Th1 cell differentiation (374), T\textsubscript{FH}-like features in CD8 T cells appear to be associated with intermediate activation/exhaustion. Thus, does it mean that T\textsubscript{FH} cells can be overstimulated to become dysfunctional? Can T\textsubscript{FH} cells become exhausted and lose their ability to help similar to what has been observed in CD8 T cells? In this context, we have shown that Fgl2 can up-regulate co-inhibitory molecules, especially TIM-3, in T\textsubscript{FH} cells. Can exposure to suppressive molecules like Fgl2 produced by T\textsubscript{FR} cells induce co-inhibitory gene modules in T\textsubscript{FH} cells similar to what IL-27 was shown to do in CD8 T cells (133) and thereby limit their ability to help and mark them for terminal dysfunction?

**Concluding Remarks**

The studies presented in this dissertation provide more insight into the ontogeny, function and regulation of T\textsubscript{FH} cells and T\textsubscript{FR} cells and the subsequent consequences on antigen-specific B cells and autoreactive B cells. We demonstrated that T\textsubscript{FR} cells highly express soluble Fgl2 and the molecule directly binds and regulates T\textsubscript{FH} cells and B cells both \textit{in vitro} and \textit{in vivo}, leading to the suppression of type 2 antibody responses. In T\textsubscript{FH} cells, Fgl2 induces the expression of Prdm1 and a panel of checkpoint molecules including PD-1, TIM-3, LAG-3 and TIGIT, potentially resulting in T\textsubscript{FH} cell dysfunction. Fgl2-deficient mice had dysregulated antibody responses at steady state and upon immunization, and mice developed autoimmunity associated with elevated autoantibodies spontaneously. The results, therefore, demonstrated Fgl2 as a novel T\textsubscript{FR} cell effector molecule that regulates humoral immunity and limits systemic autoimmunity. In addition, using single-cell RNA-seq experiments in combination with T-bet fate reporter mice, we identified Th1-like T\textsubscript{FH} cells upon NP-OVA/CFA immunization and these cells correspond to T\textsubscript{FH} cells that previously expressed T-bet. Interestingly, while T-bet fate\textsuperscript{+} T\textsubscript{FH} cells are mostly T-
bet, they still retain significant expression of Th1-associated genes at the single cell level. The results demonstrated that these Th1-like T_{FH} cells are derived from Th1 cells. Moreover, we identified STAT3 as a Th1/T_{FH} differentiation switch and its deficiency in T-bet^+ cells led to striking defect in T-bet fate^+, Th1-like T_{FH} cells. Such findings, therefore, revealed the presence of a linear differentiation trajectory of T_{FH} cells as they can be derived from another CD4 T cell subset. Taken together, these findings could provide novel therapeutic targets to intervene in order to optimize appropriate humoral effector responses in the context of vaccine design, as well as to better suppress autoreactive responses in the context of autoimmunity.
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